

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

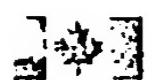
Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



Consommation et
Affaires commerciales Canada
Bureau des brevets
Ottawa, Canada
K1A 0C9

Consumer and
Corporate Affairs Canada
Patent Office

(21) (A1) 2,077,665
(22) 1991/03/04
(43) 1991/09/06

6,009,5/62

Bol. te carreg. le 250 91/13904 (43)

(51) INTL.CL. ⁵ C12N-009/64; C12Q-001/37; C07K-007/06; C07K-005/04;
A61K-037/64

(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Chymotrypsin-Like Proteases and Their Inhibitors

(72) Siman, Robert - U.S.A. ;
Nelson, Robert B. - U.S.A. ;
Kauer, James C. - U.S.A. ;
Potter, Huntington - U.S.A. ;

(73) Cephalon, Inc. - U.S.A. ;

(30) (US) 489,290 1990/03/05

(57) 44 Claims

Notice: The specification contained herein as filed

Canada

CCA 3254 (10-92) 41 7530-21-936-3254

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

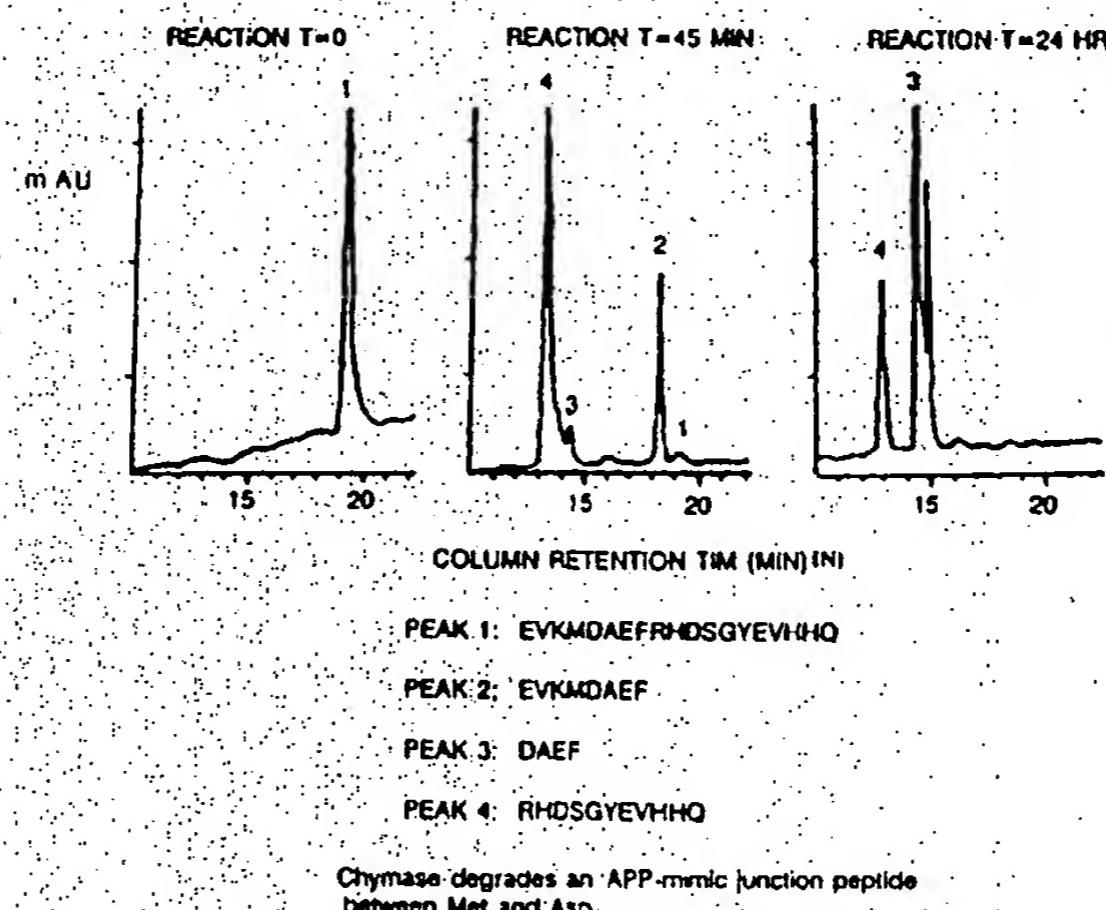
20776



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :	C07K 3/00, A23J 1/00 C12Q 1/37	A1	(11) International Publication Number: WO 91/13904 (43) International Publication Date: 19 September 1991 (19.09.91)
(21) International Application Number:	PCT/US91/01474	(74) Agent: CLARK, Paul, T.; Fish & Richardson, One Financial Center, Suite 2500, Boston, MA 02111-2658 (US).	
(22) International Filing Date:	4 March 1991 (04.03.91)	(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, PL, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.	
(30) Priority data:	489,290 5 March 1990 (05.03.90) US	(60) Parent Application or Grant (63) Related by Continuation US 489,290 (CIP) Filed on 5 March 1990 (05.03.90)	(71) Applicant (for all designated States except US): CEPHALON, INC. [US/US]; 145 Brandywine Park, West Chester, PA 19380 (US).
(72) Inventors: and (75) Inventors/Applicants (for US only):	ROBERT SIMAN [US/US]; 2646 Bittersweet Drive, Wilmington, DE 19810 (US). ROBERT NELSON, B. [US/US]; 329 Wellman Avenue, N. Chelmsford, NH 01863 (US). JAMES KAUER, C. [US/US]; 605 Willow Glen Road, Kenneth Square, PA 19348 (US). HUNTINGTON POTTER, [US/US]; 65 Park Drive, #2, Boston, MA 02215 (US).	(72) Inventors: and (75) Inventors/Applicants (for US only): SIMAN, Robert [US/US]; 2646 Bittersweet Drive, Wilmington, DE 19810 (US). NELSON, Robert, B. [US/US]; 329 Wellman Avenue, N. Chelmsford, NH 01863 (US). KAUER, James, C. [US/US]; 605 Willow Glen Road, Kenneth Square, PA 19348 (US). POTTER, Huntington [US/US]; 65 Park Drive, #2, Boston, MA 02215 (US).	(76) Published: With international search report.

(54) Title: CHYMOTRYPSIN-LIKE PROTEASES AND THEIR INHIBITORS



(57) Abstract

Disclosed is the identification, characterization, and purification of two chymotrypsin-like serine proteases characteristic of AD, called "chymase" and "multicatalytic protease". Both chymase and the multicatalytic protease have an enzymatic activity capable of cleaving between Met and Asp, which activity is required to generate beta-amyloid. Methods are provided for quantifying the activities of chymase and the multicatalytic protease in brain (and other tissues samples and fluids) to allow diagnosis of AD, and for development of inhibitors as therapeutic agents to slow progress of the disease. Novel compositions have been prepared and identified as inhibitors of the two enzymes. Substrates are also disclosed. Methods are provided for the treatment of AD and the identification of proteases capable of generating beta-amyloid protein. Methods are also provided for cloning and sequencing of human brain chymase and the multicatalytic protease, and the preparation of nucleic acid and antibody probes to them.

CHYMOTRYPSIN-LIKE PROTEASES AND THEIR INHIBITORS

Background of the Invention

This invention relates to chymotrypsin-like proteases, and substrates and inhibitors thereof, involved in the etiology of Alzheimer's disease.

Alzheimer's disease (AD) is a progressive degenerative disorder of the brain that afflicts over four million people in the United States. No effective treatment is presently available. AD is only definitively diagnosed by post-mortem histopathological methods. Even in its end-stage, AD is sometimes confused with disorders that manifest dementia, such as depression, which can be treated if correctly diagnosed. Thus, there is an enormous need for strategies to develop AD diagnostics and therapeutics.

An invariant feature of AD neuropathology is the deposition of protein, known as beta-amyloid or A4, into neuritic and cerebrovascular plaques. The presence of dense beta-amyloid plaques in large areas of the cerebral cortex is diagnostic for AD, according to guidelines of the National Institute on Aging (Khachaturian, Arch. Neurol. 42:1097, 1985). Beta-amyloid is a 39-42 amino acid peptide (Glenner et al., Biochem. Biophys. Res. Commun. 120:885, 1984; Masters et al., Proc. Natl. Acad. Sci. 82:4245, 1985). It is synthesized as part of larger precursor proteins (Kang et al., Nature 325:733, 1987; Tanzi et al., Nature 331:528, 1988; Ponte et al., Nature 331:525, 1988; Kitaguchi et al., Nature 331:530, 1988). Proteolytic processing of these beta-amyloid precursor proteins (APP) is required to generate beta-amyloid. The amino terminal of beta-amyloid is generated by cleavage of the peptide bond in APP between

- 2 -

Met⁵⁹⁶ and Asp⁵⁹⁷ (numbering according to Kang et al., supra).

Abraham et al. (Biotechnology 7:147, 1989) state that "it is interesting to note that the N-terminal of the two cleavage sites within the precursor of the beta-protein contains a methionine and could therefore result from processing by a chymotrypsin-like protease". Similarly, Van Nostrand et al. (Nature 341:546, 1989) state that "several findings have implicated the involvement of a chymotrypsin-like protease in the deposition of amyloid beta-protein in Alzheimer's disease neuritic plaques".

Abraham (Neurobiol. Aging 10:463, 1989) states that "protease inhibitors are, therefore, attractive targets for drug intervention in Alzheimer's disease".

Pope et al. (Neurochem. Res. 9:291, 1984) describe proteases found in the human brain. They state that the neuritic plaques that characterize AD are the result of excess protein accumulation consistent with impaired steady-state proteolysis and/or protein quality control. They suggest that it is likely "that comprehensive analysis of neural proteolytic machinery will be fruitful for improved understanding of the molecular and structural lesions in the cerebrospinal abiotrophies".

Among the proteases identified in mammalian brain are two serine proteases that have chymotrypsin-like activity. One is an enzyme complex referred to as "ingensin" (Ishiura et al., FEBS Lett. 189:119, 1985) or the "multicatalytic protease" (Wilk et al., J. Neurochem. 40:842, 1983). This protease complex contains chymotrypsin-like activity, in that it cleaves peptide bonds on the carboxy terminal side of aromatic or large aliphatic amino acids. Secondly, mast cells have been found in the

- 3 -

brains of several mammals (Dropp, Acta Anat. 94:1, 1976); these cells contain chymotrypsin-like protease (Woodbury et al., Proc. Natl. Acad. Sci. 75:5311, 1978; Schechter et al., J. Immunol. 137:962, 1986).

5 There are a number of publications concerning inhibitors for proteases and, in particular, for serine proteases. These include: Nakajima et al., J. Biol. Chem. 254:4027, 1979; Imperiali et al., Biochemistry 25:3760, 1986; Kettner et al., J. Biol. Chem. 259:15106, 1984; 10 Kettner et al., U.S. Patents 4,582,821, 4,499,082, 4,644,055, 4,636,492, 4,652,552, and EPA 0,187,721.

The following abbreviations are used for amino acids and amino acid residues:

	<u>AMINO ACID</u>	<u>ABBREVIATION</u>	<u>ONE LETTER CODE</u>
15	L-alanine	Ala	A
	L-arginine	Arg	R
	L-asparagine	Asn	N
	L-aspartic acid	Asp	D
	L-cysteine	Cys	C
20	L-glutamine	Gln	Q
	L-glutamic acid	Glu	E
	Glycine	Gly	G
	L-histidine	His	H
	L-isoleucine	Ile	I
25	L-leucine	Leu	L
	L-lysine	Lys	K
	L-methionine	Met	M
	L-phenylalanine	Phe	F
	L-proline	Pro	P
30	L-serine	Ser	S
	L-threonine	Thr	T
	L-tryptophan	Trp	W
	L-tyrosine	Tyr	Y

- 4 -

L-valine	Val	V
L-ornithine	Orn	
L-norleucine	Nle	
L-homoarginine	Harg	
5 L-citrulline	Cit	
L-norvaline	Nva	
L-pyroglutamic acid	Pglu	

where prefixed by "D-", the foregoing abbreviations indicate an amino acid of D-configuration.

10 The following abbreviations are used for N-terminal blocking groups:

Boc	t-Butyloxycarbonyl
Ac	Acetyl
Et	Ethyl
15 Suc	Succinyl
MeOSuc	Methoxysuccinyl
Sub	Suberyl
Ad	Adipyl
Az	Azetyl
20 DNS	Dansyl
DNP	2,4-Dinitrophenyl
Z	Benzylloxycarbonyl
Fmoc	Fluorenylmethoxycarbonyl
MeOAz	Methoxyazetyl
25 MeOAd	Methoxyadipyl
MeOSub	Methoxysuberyl

Summary of Invention

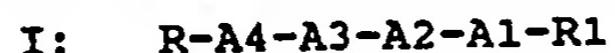
The present invention concerns the identification, characterization, and purification of two chymotrypsin-like serine proteases characteristic of AD, called "chymase" and "multicatalytic protease", that are derived from rat or

- 5 -

human brain. Both chymase and the multicatalytic protease have an enzymatic activity capable of cleaving between Met and Asp, which activity is required to generate beta-amyloid. Methods are provided for quantifying the 5 activities of chymase and the multicatalytic protease in brain (and other tissue samples and fluids) to allow diagnosis of AD, and for development of inhibitors as therapeutic agents to slow progress of the disease. Novel compositions have been prepared and identified as inhibitors 10 of the two enzymes. Methods are also provided for cloning and sequencing of human brain chymase and the multicatalytic protease, and the preparation of nucleic acid and antibody probes to them.

Thus, in a first aspect the invention features a 15 purified peptide (which term includes polypeptides and proteins) having an endopeptidase enzymatic activity which causes hydrolysis of a peptide bond between a methionine and an aspartic acid in a beta-amyloid precursor protein. By "purified" is meant that the peptide is separated from 20 components of a cell or tissue in which it naturally occurs; preferably it is purified to represent at least 70% by weight, most preferably 90%, of the peptides present in a solution or preparation.

In a second aspect, the invention features a method 25 for detection in a biological sample of an protease indicative of Alzheimer's disease. The method includes providing a substrate for the protease having the formula I:



wherein R is an N-terminal blocking group or hydrogen; R₁ 30 is a reporter group; A₄ is a covalent bond (i.e., R is directly bonded to A₃), an amino acid or a peptide, e.g., including up to about five D- or L-amino acids; A₃ a covalent bond (i.e., A₄ is directly bonded to A₂ or when A₄

- 6 -

is a covalent bond R is directly bonded to A2), or is a D-amino acid, Phe, Tyr, or Val or a conservative amino acid substituent of Val, e.g., Leu, Ile, Nle, Ala, and Nva; A2 is a hydrophobic amino acid, e.g., Leu, Ile, Nle, Phe, Val, 5 Nva, Tyr, or Pro, or preferably lysine or a conservative amino acid substituent thereof, e.g., Arg, Orn, Cit, and Harg or when A4 includes at least two amino acids, A2 is any amino acid; and A1 is Met, Phe, Nle, Leu, Ile, Tyr, or 10 3-phenylproline; when A4 and/or A3 are a covalent bond, R may be an alkane dicarboxylic acid or alkane carboxylic acid of 2 to 20 carbon atoms, or a lower alkyl monoester thereof, optionally substituted by an alkyl, aralkyl, or aryl substituent; preferred substituents are isopropyl, isobutyl, benzyl, or phenyl, and preferred lengths are 3 to 10 carbon 15 atoms. The method also includes contacting the biological sample with the substrate, and detecting cleavage of the substrate as an indication of the presence of the protease in the biological sample.

R1 is a reporter group capable of forming an amide 20 or ester linkage with the C-terminal carbonyl of amino acid residue A1 and which, on release from the peptide sequence by the cleavage reaction, forms a molecule R1-H or an anion R1⁻ which may be detected by visual or instrumental means.

The precise nature of the reporter group R1 is not 25 critical to the present invention. By "reporter group" is meant any chromogenic, magnetic, fluorogenic, luminescent, antigenic, stable free radical or radioactive group which is detectable by any one or more of the techniques used for detecting particular analytes when in solution, or attached 30 to a suitable surface.

When R1 is a chromogenic group, it forms a covalent molecule R1-H after cleavage, or a covalent ion R1⁻, which

- 7 -

may optionally be separated from the unreacted peptide by solvent extraction, precipitation, or other chemical or physical means which allows the free R₁-H or R¹⁻ to be determined visually or instrumentally. For example, when 5 R₁-H is p-nitroaniline (R¹⁻ is a p-nitroanilide (pNa) ion), it may be determined quantitatively by its ultraviolet or visible light absorption in a photometer, or visually by comparison with a series of standard solutions. Other suitable chromogenic groups include nitronaphthyloxy, naphthylamino, nitronaphthylamino, and quinolinamino.

10 When R₁ is a fluorogenic group, the free R¹⁻ or R₁-H may be detected in solution by exposing the solution to light of one wavelength and detecting fluorescence at another, usually longer, wavelength. Suitable fluorogenic 15 groups include 4-methylcoumarin-7-amino (AMC), 2-naphthylamino, 4-trifluoromethylcoumarin-7-amino, and 4-methylumbelliferyl.

R₁-H may be measured quantitatively by nuclear magnetic resonance (NMR) by incorporating a substituent with 20 a magnetically distinctive nucleus which produces a sharp resonance detection, and a fluoroalkyl or fluoroaryl substituent for ¹⁹F magnetic resonance detection.

When R₁ is a stable free radical (e.g., nitroxyl or diphenylpicrylhydrazyl) substituent this permits its 25 detection by electron spin resonance (ESR).

If cleavage of R₁ from the peptide is accompanied by a marked change in the physical properties of the R₁ group, e.g., a significant change in the intensity or wavelength of the ultraviolet absorption maximum, the physical separation 30 of the released substituent (R¹⁻ or R₁-H) from the unreacted substrate may optionally be omitted. The amount of cleavage is determined quantitatively by measurement of the

- 8 -

absorption of the released substituent R1-, at or near its ultraviolet or visible light absorption maximum.

By "N-terminal blocking group" is meant a D-amino acid or an arylcarbonyl, alkylcarbonyl, alkoxy carbonyl, 5 aryloxycarbonyl, aralkyloxycarbonyl, aralkylsulfonyl, alkylsulfonyl, or arylsulfonyl peptide protecting group, or other equivalents known to those skilled in the art of peptide synthesis and which are known to protect molecules from degradation by aminopeptidases (Gross and Meienhofer, 10 eds., The Peptides, vol. 3, Academic Press, New York, 1981 pp. 3-81, describes numerous suitable amine protecting groups). As used herein, either individually or as part of a larger group, "alkyl" means a linear, cyclic, or branched-chain aliphatic moiety of 1 to 20 carbon atoms; 15 "aryl" means an aromatic moiety, e.g., phenyl, of 6 to 18 carbon atoms, unsubstituted or substituted with one or more alkyl, substituted alkyl, nitro, alkoxy, or halo groups; "substituted alkyl" means an alkyl group having a substituent containing a heteroatom or heteroatoms such as 20 N, O or S; "halo" means Cl or Br, and "alkaryl" means an aryl moiety of 7 to 19 carbons having an aliphatic substituent, and, optionally, other substituents such as one or more alkyl, substituted alkyl, alkoxy or amino groups. "Aralkyl" means a linear or branched chain aliphatic moiety 25 of 7 to 18 carbon atoms including an aryl group or groups.

Examples of suitable N-terminal blocking groups include formyl, acetyl, trifluoroacetyl, benzyloxycarbonyl (carbobenzyloxy), substituted benzyloxycarbonyl, tertiary butyloxycarbonyl, isopropylloxycarbonyl, allyloxycarbonyl, 30 phthaloyl, benzoyl, acetoacetyl, chloroacetyl, phenoxy carbonyl, methoxysuccinyl, succinyl, adipyl, suberyl, 2,4-dinitrophenyl, dansyl, p-methoxybenzenesulfonyl,

- 9 -

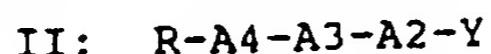
p-toluenesulfonyl, methanesulfonyl, D-serine, and D-glutamic acid.

In preferred embodiments, the biological sample is isolated from brain, liver, heart, muscle, white blood 5 cells, mast cells or fibroblasts, serum or cerebrospinal fluid; the protease is chymase; the protease is multicatalytic protease.

In a third aspect, the invention features a substrate for a protease, having the formula I described 10 above wherein R is an N-terminal blocking group or hydrogen; R1 is a reporter group; A4 is a covalent bond (i.e., R is directly bonded to A3), an amino acid or a peptide, e.g., including up to about five D- or L-amino acids; A3 a 15 covalent bond (i.e., A4 is directly bonded to A2 or when A4 is a covalent bond R is directly bonded to A2), or is a D-amino acid, Phe, Tyr, or Val or a conservative amino acid substituent of val, e.g., Leu, Ile, Nle, Ala, and Nva; A2 is 20 a hydrophobic amino acid, e.g., Leu, Ile, Nle, Phe, Val, Nva, Tyr, or Pro, or preferably lysine or a conservative amino acid substituent thereof, e.g., Arg, Orn, Cit, and Harg or when A4 includes at least two amino acids, A2 is any 25 amino acid; and A1 is Met, Phe, Nle, Leu, Ile, Tyr, or 3-phenylproline; when A4 and/or A3 are a covalent bond, R may be an alkane dicarboxylic acid or alkane carboxylic acid of 2 to 20 carbon atoms, or a lower alkyl monoester thereof, 30 optionally substituted by an alkyl, aralkyl, or aryl substituent; preferred substituents are isopropyl, isobutyl, benzyl, or phenyl, and preferred lengths are 3 to 10 carbon atoms. In preferred embodiments, A3 is Val, Nle, Nva, Ile, Leu, Tyr, or Phe; A2 is Lys, Arg, Orn, Cit, or Harg; A4 is Glu or Asp; R is Boc, Fmoc, Ac, Et, Suc, MeOSuc, Az, MeOAz, Ad, MeOAd, Sub, MeOSub, DNS, DNP, Z, or a D-amino acid; and R2 is pNa or AMC.

- 10 -

In a fourth aspect, the invention features an inhibitor of a protease, having the formula II:



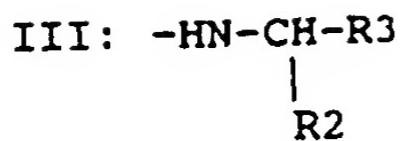
where R, A4, A3, and A2 are as described above, and Y is a group reactive with the active site of the protease. These inhibitors have therapeutic utility derived from their ability to inhibit the enzyme "chymase" or the enzyme "multicatalytic protease" or related enzymes. These inhibitors include peptide sequences protected at their N-terminus by a protecting group, and attached at their C-terminus by a covalent bond to a moiety Y, which interacts strongly with functional groups located near the reactive site of the enzyme.

The N-terminal blocking group provides metabolic stability to the composition by reducing or eliminating its ability to be degraded by endogenous aminopeptidases. The carboxyl terminal moiety Y allows a strong chemical interaction, mediated by covalent or noncovalent bond formation, at the active site of the enzyme to effectively inactivate the enzyme.

Y is a group which strongly interacts with functional groups in or near the enzyme active site and is covalently attached to the carbonyl group of amino acid A2 by an amide bond. It is provided with a lipophilic side chain substituent R2, which interacts with the lipophilic substituent recognition site of the enzyme to be inhibited, e.g., chymase or the multicatalytic protease, and with a functional group R3, which can react with or interact with an adjoining serine hydroxyl group to form a transition-state analog (Rich, Protease Inhibitors, ed. Barrett and Salversen, Elsevier, 1986, p. 167).

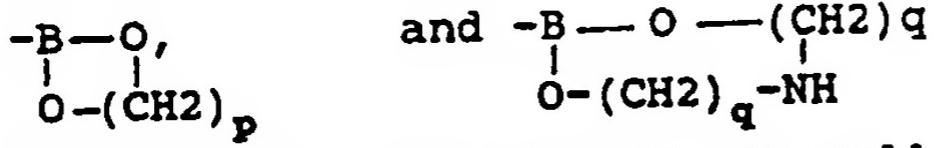
Y may be derived from an amino acid analog Y-H in which Y has the formula III:

- 11 -



wherein R₂ may be a saturated or unsaturated alkyl or
5 substituted alkyl group of 2 to 10 carbon atoms, alicyclic or aromatic groups of 5 to 10 carbon atoms or arylalkyl groups of up to 11 carbon atoms. Examples of suitable R₂ groups include ethyl, n-propyl, isopropyl, n-butyl, sec-butyl, isobutyl n-pentyl, n-hexyl, isohexyl, n-decyl, 10 phenyl, benzyl, beta-phenylethyl, alpha-naphthyl, beta-naphthylmethyl, cyclohexyl, cyclohexylmethyl, adamantyl, alpha-styryl, beta-styryl, and propargyl.

Examples of suitable R₃ groups include the aldehyde and ketone functions -CHO, -CO-CH₃, -CO-CH₂Cl, -CO-CH₂Br, 15 and boronic acid residues, e.g., -B-(OH)₂,



where p and q are 2 or 3; as well as alphadiketones, e.g., -C-C-CH₃, and alphaketoesters, e.g., -C-C-OCH₃. 20



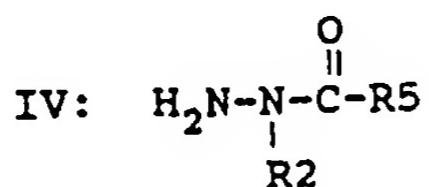
Other suitable R₃ groups include -SO₂F, -C₆H₄-SO₂F, and -PO(OR₄)F where R₄ is a lower alkyl or aralkyl substituent.

Additional suitable Y groups include the acylating 25 heterocyclic ring systems described by Powers and Harper, in Proteinase Inhibitors, A.J.Barrett and G. Salversen, eds., Elservier, New York, 1986, pp. 108-132. These substituents generally interact with the enzyme active-site serine and may subsequently react with other adjoining functionalities 30 such as histidine side chains. Examples of such Y groups include N-substituted saccharins, as well as derivatives of benzoxazin-4-ones, 3-alkoxy-4-chloroisocoumarins, oxazine-2,6-diones, substituted isatoic acids,

- 12 -

halomethylcoumarins, N-nitrosoisoquinolinones, haloenol lactones, isobenzofuranones, ynenol tetrahydro-2-furanones, 2-pyanones, chloropyrones, chloroisocoumarins and the like.

Other Y groups may be derived from alpha-aza amino acids and their amide, ester, and peptide derivatives of formula IV:



where R2 is as defined in formula III and R5 may be derived respectively from an amine or ammonia, an alcohol, or a peptide sequence substituted at its amino terminus. Examples of the synthesis of such aza amino acids and azapeptides are reviewed by J. Gante, *Synthesis*, 405-413, 1989. Preferred values of R5 are amino acids or peptide sequences found at the amino terminus of beta-amyloid, e.g., asp, asp-ala, asp-ala-glu, asp-ala-glu-phe and their esters and amides.

In preferred embodiments, Y has the formula:

$\text{H}_3\text{N}-\text{CH}-\text{R}3,$

R2

where R2 is a lipophilic side chain substituent and R3 is a functional group which reacts with a hydroxyl group of the protease to form a transition-state analog; R2 is a saturated or unsaturated alkyl or substituted alkyl group which includes two to ten carbon atoms or an alicyclic or aromatic group comprising five to ten carbon atoms or an arylalkyl group which includes eleven or fewer carbon atoms; R2 is an ethyl, n-propyl, isopropyl, n-butyl, or sec-butyl group; R3 is -CHO, -COCH₃, COCH₂Cl, or COCH₂Br; the protease is chymase; the protease is multicatalytic protease.

2077655

- 13 -

In a fifth aspect, the invention features methods for purifying a protease to at least 70%, preferably 90% or greater, purity from a biological sample. One method includes homogenizing the biological sample, e.g., a 5 biological sample from a mammal such as from human, rat, cow, or pig, in a low salt buffer including detergent; isolating the particulate fraction from the homogenized tissue; dissolving the particulate fraction in a high salt buffer; separating the soluble fraction from the high salt 10 buffer; binding the protease to, and then eluting it from, an ion exchange or a heparin column; and binding the protease, and then eluting it from, an affinity column.

In preferred embodiments, the biological sample is isolated from brain, liver, heart, skeletal muscle, white 15 blood cells, mast cells or fibroblasts; the low salt includes less than 0.5M salt; the detergent is ionic or nonionic, e.g., Triton X-100; the particulate fraction is separated from the homogenized tissue by centrifugation, e.g., at between 30,000 to 300,000 x g for 15 to 120 20 minutes; the high salt is greater than 0.5M salt; the soluble fraction is separated from the high salt by centrifugation, e.g., at 30,000 to 300,000 x g for 15 to 120 minutes; the protease is bound to a heparin column; the protease is chymase; the protease is multicatalytic 25 protease; and the affinity column includes a substrate for the enzyme. In this affinity column the amino terminus of the substrate is attached to the carrier column material by a covalent bond, preferably by an amide bond; most preferably the substrate has the formula V:

30 V: W-X-Y-Z

where W is deleted, an amino acid or a peptide, e.g., including up to about five D- or L-amino acids; X is deleted, or is Phe, or is Val or a conservative amino acid

- 14 -

substituent of Val, e.g., Leu, Ile, Nle, Ala, and Nva; Y is a hydrophobic amino acid, e.g., Leu, Ile, Nle, Phe, Val, Nva, Pro, or Lys or a conservative amino acid substituent of Lys, e.g., Arg, Orn, Cit, and Harg, or, when W includes at least two amino acids, Y may be any amino acid; and Z is Met, Phe, Nle, Leu, Ile, Tyr or 3-phenylproline.

Alternatively, when W is deleted, X may be a deleted or may be an omega-aminoalkanecarboxylic acid of 2 to 20 carbon atoms in chain length, optionally substituted at the alpha or beta position by an alkyl, aralkyl, or aryl substituent. Preferred substituents are isopropyl, isobutyl, benzyl, and phenyl, and preferred chain lengths are 2-8 carbon atoms.

The second method includes homogenizing the tissue in a low salt buffer; isolating the soluble fraction from the homogenized tissue; binding the protease to, and then eluting it from, an ion exchange or heparin column; concentrating the protease and passing it through a gel filtration column; binding the protease, and then eluting it from, an hydroxyapatite or heparin column.

In a sixth aspect, the invention features a method for treating Alzheimer's disease. The method includes providing an inhibitor of formula II as described above, and administering the inhibitor to a patient in a pharmaceutically acceptable carrier.

In preferred embodiments, the administration is directly into the brain; the administration is intramuscular, oral, or intranasal; the protease inhibitor is an inhibitor of chymase; the protease inhibitor is an inhibitor of multicatalytic protease.

In a seventh aspect, the invention features a method for screening candidate chymase inhibitors. The method includes the steps of: (a) incubating a chymase substrate

2077065

- 15 -

(e.g., a substrate corresponding to formula I above) with one or more candidate chymase inhibitors and chymase; and (b) determining whether the one or more candidate chymase inhibitors decrease the rate of cleavage of the substrate by the chymase.

In an eighth aspect, the invention features a method for screening candidate multicatalytic protease inhibitors. The method includes the steps of: (a) incubating a multicatalytic protease substrate (e.g., a substrate corresponding to formula I above) with one or more candidate multicatalytic protease and multicatalytic; and (b) determining whether the one or more candidate multicatalytic protease inhibitors decrease the rate of cleavage of the substrate by the multicatalytic protease.

In an eighth aspect, the invention features a method for screening candidate proteases capable of cleaving a beta-amyloid precursor protein between Met and Asp. The method includes of: (a) providing a peptide having the sequence: EVKMDAEFRHDSGYEVHHQ; (b) contacting the candidate protease with the peptide; and (c) determining whether the candidate protease cleaves the substrate.

In a ninth aspect, the invention features a method for screening proteases capable of cleaving a beta-amyloid precursor protein between Met and Asp. The method includes the steps of (a) providing a substrate having the sequence R-Glu-Val-Lys-Met-R1, wherein R is hydrogen or an N-terminal blocking group, R1 is a reporter group; (b) contacting the candidate protease with the substrate; and (c) determining whether said candidate protease cleaves the substrate.

In a tenth aspect, the invention features a method for treating a patient afflicted with Alzheimer's disease. The method includes administering to the patient an

- 16 -

inhibitor of chymase in a pharmaceutically acceptable carrier.

In an eleventh aspect, the invention features a method for treating a patient afflicted with Alzheimer's disease. The method includes administering to the patient an inhibitor of multicatalytic protease in a pharmaceutically acceptable carrier.

By "inhibitor of chymase" is meant a molecule capable of decreasing the ability of chymase to catalyze proteolytic cleavage of a protein, particularly proteolytic cleavage of a beta-amyloid precursor protein.

By "inhibitor of multicatalytic protease" is meant a molecule capable of decreasing the ability of chymase to catalyze proteolytic cleavage of a protein, particularly proteolytic cleavage of a beta-amyloid precursor protein.

This invention provides enzymes able to cleave a Met-Asp bond in an APP. The quantity of such enzymes in tissue is diagnostic of AD; their detection allows a rapid, simple, and non-invasive assay for AD. Such detection may be by enzymatic test, Western blot, or Southern or Northern blotting techniques. Further, inhibition of the enzyme activity *in vivo* allows progression of AD to be slowed. Such inhibition is caused by the above described inhibitors, and by antibodies to the enzymes.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

30 Drawings

Fig. 1 depicts an SDS-polyacrylamide gel analysis of purified chymase;

- 17 -

Fig. 2 depicts the results of the purification scheme for brain chymase;

Fig. 3 is a depiction of a comparison of the partial amino acid sequence of purified brain chymase (Brain) and 5 the partial amino acid sequence of rat mast cell protease I (RMCP I);

Fig. 4 is a set of graphs which depict the results of column chromatography used to purify chymase;

Fig. 5 is a schematic illustration of the beta/A4 10 protein and the region of beta/A4 protein used in design protease substrates;

Fig. 6 is a set of graphs which depict the degradation of substrates brain chymase (panel A) chymotrypsin (panel B), and cathepsin G (panel C);

15 Fig. 7 is a set of graphs which illustrate the results of HPLC analysis of peptides generated by chymase degradation;

Fig. 8 depicts an SDS-polyacrylamide gel analysis purified chymase;

20 Fig. 9 depicts the results of a purification scheme for chymase;

Fig. 10 depicts the results of column chromatography used to purify chymase;

25 Fig. 11 depicts the results of kinetic analysis of degradation of substrates by rat brain chymase;

Fig. 12 is a set of graphs which depict the inhibition of protease degradation by inhibitors;

30 Fig. 13 depicts inhibition of various proteases by inhibitors;

Fig. 14 depicts the effect of inhibitors of degradation of substrates by a protease;

Fig. 15 is a graphical representation of protease activity in various parts of a Alzheimer's disease brain;

- 18 -

Fig. 16 depicts the structures of a number of protease inhibitors.

Substrates and Inhibitors

Substrates useful in this invention have the general formula I described above. They can be used to measure the level of chymotrypsin-like proteases characteristic of Alzheimer's disease in body tissues and fluids. These compositions are peptide sequences preferably protected at their amino termini by a blocking group and attached at the carboxy termini by an amide or ester bond to a reporter group which is released when the bond is cleaved by the protease. Such peptide compositions also include salts of the above general formulae. Generally, the amino acid analogs are chosen such that their identity and sequence substantially corresponds to, or is identical to, those amino acids located immediately adjacent to and upstream of a cleavage recognition site of the enzymes chymase or the multicatalytic protease in an APP.

By "upstream" is meant the direction which is the reverse of the direction of translation, i.e., toward the amino terminus of a peptide.

By "substantially corresponds" is meant that a peptide sequence comprises a like number of amino acid residues as a reference sequence, the identity and sequence of which are exactly homologous, or conservatively substituted, in relation to the reference sequence. By "conservatively substituted" is meant that a given amino acid residue is replaced by a biologically similar residue. Examples of conservative substitution include substitution of one alkyl or substituted alkyl residue for another such as Nle, Met, Ile, Val, Leu, Phe or Tyr for one another, or substitution of one polar residue for another, such as Lys

- 19 -

and Arg; Glu and Asp; or Gln and Asn. "Residue" means either an amino acid or amino acid analogue.

Acceptable salts of the compounds include the salts of free peptide acids including sodium, potassium, magnesium, calcium, and ammonium salts as well as acid addition salts of the free peptide bases including both inorganic and organic acids such as hydrochloric, sulfuric, phosphoric, acetic, citric, or trifluoroacetic acids.

Preferred compounds of this invention include peptide derivatives of formula I in which A1 is selected from the group Phe, Met, Leu, Ile, Nle, Tyr, or 3-phenylproline; A2 is selected from the group Lys, Arg, Orn, Cit, or Harg; A3 is selected from the group Val, Leu, Ile, Nle, Phe, Nva, Pro, or is a D-amino acid, or a covalent bond (i.e., A4 is bonded directly to A2 or when A4 is a covalent bond R is bonded directly to A2); A4 is Glu, Asp, D-Glu or D-Asp, or is a covalent bond (i.e., R is bonded directly to A3); R is one of the protecting groups previously described or a D-amino acid; and R1 is selected from pNa and AMC. Specific examples of such compounds are shown in Figures 11 and 13.

Inhibitors have the general formula II described above. These are designed in a manner similar to the substrates but have a reactive C-terminal group as described above. Specific examples of such inhibitors include those shown in Figures 12, 13 and 16.

The above compositions can be used to measure the levels of the enzymes chymase and the multicatalytic protease in body tissues and fluids and to inhibit their activity. These compositions are generally peptide or peptidomimetic sequences protected at the amino terminus by a blocking group. These compounds can be prepared by the reaction of a precursor peptide derivative, for example

- 20 -

R-A4-A3-A2-OH with the amino acid derivatives H-A1-R1 or H-Y respectively.

The peptide precursors may be prepared by well-known synthetic procedures using either the solution phase
5 techniques described in "The Peptides: Analysis, Synthesis,
Biology" Volume I (1979), eds. Gross et al. (eds.), Academic
Press, or the solid phase techniques described in Volume II
(1980) of the above series.

The peptide precursors R-A4-A3-A2-OH with protecting
10 groups in place may be prepared by the solid phase method
using the support resin described by Mergler et al.,
Tetrahedron Lett. 29:4009 (1988) and commercially available
under the name "Sasrin" from Bachem Biosciences, Inc.,
Philadelphia, PA. 19104. The peptide sequences are built up
15 on the resin by sequential treatment of the resin with amino
acid derivatives beginning with the fluorenylmethoxycarbonyl
derivative of amino acid A2 (Fmoc-A2), in the presence of a
coupling reagent such as benzotriazolyloxytris
(dimethylamino) phosphonium hexafluorophosphate (BOP),
20 followed by treatment with piperidine to remove the Fmoc
protecting group as described by Atherton et al., J. Chem.
Soc. Perkin Transactions I, 2057 (1985). The use of the BOP
reagent is described by Castro et al., Synthesis, 751-2
(1976).

25 Subsequently the Fmoc derivative of A3 and the
corresponding components of R-A4 (see below) are added with
Fmoc deblocking by piperidine between successive amino acid
additions. The functional side chains of the amino acids
A2, A3, and A4 are protected as follows: lysine is protected
30 by the t-butoxycarbonyl (Boc) group; histidine and cysteine
are protected by the N-triphenylmethyl (trityl) group;
arginine is protected with the
4-methoxy-2,3,6-trimethylphenylsulfonyl (Mtr) group;

- 21 -

tyrosine, serine, and threonine are protected as the t-butyl ethers (t-Bu); and glutamic and aspartic acids are protected as the t-butyl esters (Ot-Bu). These particular protecting groups remain on the peptide-resin complex throughout the 5 solid phase synthesis of peptide R-A4-A3-A2 on the resin as well as during the cleavage of the peptide precursor R-A4-A3-A2-OH from the resin with a dilute solution of trifluoroacetic acid (TFA) in methylene chloride.

Alternatively, this peptide precursor may be built 10 up by solution phase methods using the strategies described in Gross and Meienhofer, supra (volume I). The solution phase strategy is particularly well suited to shorter peptides in which A4 is one or two amino acids. Thus, where A4 is a single amino acid, a peptide is built up by reacting 15 the Fmoc derivative of A4 as a N-hydroxysuccinimide ester (Fmoc-A4-OSu) with a buffered solution of amino acid A3. The resulting Fmoc-A4-A3-OH can next be reacted with the benzyl ester of amino acid A2 (A2-OBzl) to give Fmoc 20 A4-A3-A2-OBzl. Alternatively, Fmoc-A4-OSu can be reacted directly with the dipeptide ester A3-A2-OBzl to give the same product.

Fmoc-A4-A3-A2-OBzl is next treated with piperidine to remove the Fmoc protecting group, and can be reacted with 25 R-A5 (where A5 is an optional amino acid, see below) in the presence of BOP to give R-A5-A4-A3-A2-OBzl. Alternatively, Fmoc-A3-A2-OBzl can be prepared and reacted to give R-A4-A3-A2-OBzl using this methodology.

The terminal group R-A5 (or R-A4) may be prepared by treatment of the side-chain-protected terminal amino acid 30 group A5-OH with an acylating derivative of R. Thus when R is an acyl (e.g., alkylcarbonyl, arylcarbonyl, alkoxy carbonyl, aryloxycarbonyl, or aralkyloxycarbonyl) or a sulfonyl (e.g., arylsulfonyl, alkylsulfonyl, or

- 22 -

aralkylsulfonyl) function, the group R may be attached to the side-chain-protected group A5-OH using the acid chloride of R (R-Cl), or the symmetric anhydride of R (R-OR), or the mixed carbonic anhydride of R (e.g., R-O-COO-isobutyl) or 5 preferably an active ester of R such as its N-hydroxysuccinimide ester in the presence of a pH-buffering agent such as sodium bicarbonate or N-methylmorpholine.

When the N-terminal group R is a D-amino acid it may most conveniently be added to the side-chain-protected amino 10 acid function A5-OH as the Boc derivative of R to give Boc-R-A5-OH. The Boc protecting group is then carried throughout the synthesis of the protected epeptides and is removed at the end by treatment with TFA.

Side-chain-protected Fmoc-A4-A3-A2-OBzl prepared as 15 described above is next deblocked with piperidine to give A4-A3-A2-OBzl which is, in turn, condensed with side-chain-protected R-A5-OH using BOP or diisopropyl carbodiimide to yield R-A5-A4-A3-A2-OBzl. Chemical reduction or catalytic hydrogenation of this latter compound 20 gives the side-chain-protected precursor peptide derivative.

This side-chain-protected precursor peptide is then converted to an active ester or a mixed anhydride (Gross and Meienhofer, I, loc. cit.) which in turn is reacted with H-Y to yield the inhibitors of formula II.

25 The substrate compounds H-A1-R1 are purchased or are prepared by treatment of an active ester of Boc-A1 with H-R1 followed by treatment of the resulting Boc-A1-R1 with hydrochloric acid or TFA. Thus Boc-Phe hydroxysuccinimide (Fluka Chemical Corp., Ronkonkoma, N.Y.) is treated with 30 p-nitroaniline to give Boc-Phe-p-nitroanilide. Treatment of this with TFA gives Phe-p-nitroanilide.

The compounds H-Y are synthesized by methods well documented in the literature:

- 23 -

Where H-Y is an alpha amino aldehyde (i.e., R₃ is CHO), the corresponding methyl, ethyl or butyl acetal is prepared as described by Gacek et al., Tetrahedron 30, 4233 (1974) or by Edwards, European Patent Application 291234

5 filed May 5, 1988.

Where H-Y is a chloromethyl ketone (i.e., R₃ is COCH₂Cl) the hydrochloride salt of the chloromethyl ketone may be purchased (Bachem Bioscience, Inc., Philadelphia, PA.) or can be prepared by conversion of the Boc-protected 10 amino acid to a mixed carbonic anhydride followed by reaction with diazomethane to give diazoketones, then with anhydrous hydrogen chloride (Kettner et al., Arch. Biochem. Biophys. 162:56 (1974) and Fittkau, J. Prakt. Chem. 315:1037, 1973). The corresponding bromomethyl ketones 15 (i.e., R₃ is COCH₂Br) may be prepared similarly by the substitution of hydrogen bromide for hydrogen chloride (C. Kettner et al., supra). The corresponding methyl ketones are prepared by hydrogenolysis of the chloromethyl ketones with palladium-on-charcoal in methanol (Kettner et al., 20 U.S. Patent 4,652,552).

Where H-Y is a boroamino acid (i.e., R₃ is B(OH)₂) derivative, the cyclic ester of the boroamino acid is prepared substantially as described by Shenvi, U.S. Patent 4,537,773 and after coupling to the precursor peptide may 25 optionally be converted to the free peptide boronic acid as described by Shenvi and Kettner in U.S. Patent 4,499,082 and J. Biol. Chem. 259:15106 (1984).

Where H-Y is an alphadiketone or alpha-keto ester (i.e., R₃ is COCOCH₃ or COCOOC₂H₅) the requisite amino 30 diketones or alpha-keto esters may be prepared as described by Angelastro et al., J. Med. Chem. 33:13 (1990) and references therein.

- 24 -

Where H-Y is a trifluoromethyl ketone (i.e., R₃ is COCF₃) the requisite amino ketones may be prepared as described by Imperiali and Abeles, Biochemistry 25:3760 and supplementary pages (1986).

5 The amino-terminal substituents W and R in formulas II, III and V are derived from the corresponding carboxylic acids W-OH and R-OH which may be purchased commercially (e.g., from Narchem Corporation, Chicago, IL) or prepared as described in the following paragraphs using
10 techniques familiar to those skilled in the art.

The aminoalkanecarboxylic acids (W-OH as required for formula V compounds) may be prepared by sequential alkylation of ethyl malonate by an alkyl or arylalkyl bromide followed by an omega-phthalimido-alpha-bromoalkane.
15 The resulting substituted malonic ester may be hydrolyzed by mild base and decarboxylated by treatment with mild acid to yield an omega-phthalimido-alpha-alkyl (or arylalkyl)-substituted alkanecarboxylic acid. This intermediate may be chain extended by the Arndt Eistert homologation reaction
20 (Eistert in "Newer Methods in Preparative Organic Chemistry", 1:513). Finally, strong base hydrolysis is used to remove the phthalimido protecting group to give W-OH.

25 The substituted alkane carboxylic acids (R-OH) as required in formulas I and II may similarly be prepared by the sequential alkylation of ethyl malonate with alkyl or arylalkyl bromides follow as above by mild basic hydrolysis and mild acid decarboxylation. The resulting alpha-substituted alkane carboxylic acid may be extended as above by the Arndt-Eistert homologation reaction.

30 The omega monoesters of substituted alkanecarboxylic acids (R-OH as required in certain cases in formulas I and II) may be prepared by the sequential alkylation of ethyl

2077865

- 25 -

malonate with an alkyl or arylalkyl bromide followed by alkylation with the methyl or ethyl ester of an omega-bromo (or -chloro) alkane carboxylic acid. Followed by mild acid hydrolysis and decarboxylation the resulting alpha-
5 substituted alkane dicarboxylic acid omega-ester may be extended as above by the Arndt-Eistert homologation reaction.

The resulting racemic carboxylic acids (W-OH and R-OH) may be resolved into their enantiomeric forms by passage
10 through a chiral chromatographic column or by conversion to diastereoisomeric salts of asymmetric bases such as quinine, cinchonine, brucine and the like followed by fractional crystallization.

Example 1: Preparation of Precursor Peptides

15 These compounds were prepared by solution phase methods using N-hydroxysuccinimide esters (Anderson et al., J. Am. Chem. Soc. 86:1839, 1964) or mixed carbonic anhydrides (Anderson et al., J. Am. Chem. Soc. 89:5012, 1967).

20 The N-hydroxysuccinimide esters were prepared by treatment of a solution of 2 grams of the protected amino acid or peptide in 25 ml of dioxane with a molecular equivalent of N-hydroxysuccinimide and a molecular equivalent of diisopropylcarbodiimide (DIPCDI). The
25 solution was stirred overnight at room temperature, and solvent was then evaporated under reduced pressure. The residue was dissolved in ethyl acetate, washed with water, 5% aqueous citric acid, and again with water. The mixture was dried ($MgSO_4$), filtered, and the solvent removed under reduced pressure on a rotary evaporator. The purity of the
30 resulting solid product was verified by thin layer chromatography. From 2.0g of Fmoc-Val, 0.75g of

- 26 -

N-hydroxysuccinimide and 0.70 ml of DIPCDI was obtained 2.6g of Fmoc-Val-N-hydroxysuccinimide ester (Fmoc-Val-Osu). 6.5g of succinic acid monomethyl ester and 6.1g of N-hydroxysuccinimide and 6.5 ml of DIPCDI in 60 ml of dioxane yielded 8.4g of Methoxysuccinyl N-hydroxysuccinimide ester (MeOSuc-Osu). In the last case it was found that the water washing caused a significant loss of product, thus minimum quantities of water were employed.

These N-hydroxysuccinimide esters belong to a group of compounds called active esters which react readily with the primary or secondary amino groups of amino acids in aqueous or partially aqueous solution to yield peptides in high yield as follows:

a) MeOSuc-Glu(t-Bu)

A solution of 1.0g glutamic acid-gamma-t-butyl ester in 5 ml water and 0.4 ml triethylamine was treated with a solution of 1.1g MeOSuc-Osu in 10 ml dioxane. The solution was stirred for 3 hours and the organic solvents were removed under reduced pressure on a rotary evaporator. The residue was taken up in 50 ml ethyl acetate and the resulting solution was washed once with water, three times with 5% aqueous citric acid, and finally with water. The organic layer was dried ($MgSO_4$), and the solvent was removed under reduced pressure. The residual, pale yellow oil, was dissolved in ether and 400 μ ls dicyclohexylamine was added. The resulting crystalline precipitate was filtered off and washed with small portions of ether and petroleum ether. The yield of N-Methoxysuccinyl glutamic acid gamma-t-butyl ester (MeOSuc-Glu (t-Bu)) was 0.9 gram.

b) Fmoc-Val-Lys (Boc)

A suspension of 1.0g epsilon-t-butyloxycarbonyl lysine in 10 ml water and 0.40 ml N-methylmorpholine (NMM)

- 27 -

was treated with a solution of 1.3g Fmoc-Val-Osu in 10 ml dioxane. The initially cloudy suspension became clear after stirring overnight. The resulting solution was treated with 20 ml 2% aqueous sodium bicarbonate solution and was twice extracted with ether. The ether extracts were discarded, and the aqueous solution acidified with dilute hydrochloric acid to pH 1.5 and extracted with three 75 ml portions of ethyl acetate. The combined extracts were washed once with water and dried ($MgSO_4$), and solvent was removed under reduced pressure to yield 1.2g solid Fmoc-Val-Lys(Boc).

10 c) MeOSuc-Glu(t-Bu)-Val-OBzl

A solution of 11.6g Fmoc-Glu(t-Bu)-Val-OBzl in 50 ml methylene chloride was treated with 20 ml piperidine and stirred for 30 minutes at room temperature. Solvent was removed under reduced pressure and remaining traces of piperidine removed by the repeated addition and evaporation of ethyl acetate portions. The residual gamma-t-butyl-glutamyl valine benzyl ester was a pale yellow solid. It was dissolved in 30 ml of a 1:1 mixture of dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) and mixed with a solution of the monomethyl succinate acid N-hydroxysuccinimide ester obtained from 40 millimoles (5.28g) of methoxysuccinic acid. To this solution 2.6 ml NMM was added, and the reaction mixture stirred at room temperature overnight. Ethyl acetate (250 ml) was added, and the resulting solution extracted once with 100 ml water, three times with 100 ml 3% citric acid, three times with 100 ml 2% sodium bicarbonate and finally with 100 ml water. The organic layer was dried ($MgSO_4$) and evaporated to yield 7.8 grams of pale yellow solid N-methoxysuccinyl gamma-t-butylglutamyl valine benzyl ester.

30 d) MeOSuc-Glu(t-Bu)-Val-Osu

- 28 -

A solution of 7.6g MeOSuc-Glu(t-Bu)-Val-OBzl in 60 ml acetic acid was mixed with 2g 10% palladium-on-carbon in 50% water and hydrogenated at 40 p.s.i. for 3.5 hours. The product was filtered, and solvent 5 evaporated to give 7.1g of sticky solid methoxysuccinyl gamma-t-butyl-glutamyl-valine. This product was dissolved in 60 ml tetrahydrofuran (THF), and 2.3g N-hydroxysuccinimide and 2.6 ml DIPCDI added. The reaction mixture was stirred overnight and solvent then evaporated. 10 The residue was stirred with 200 ml ethyl acetate and the precipitated diisopropyl urea filtered off and discarded. The filtrate was washed with 100 ml water, 2% sodium bicarbonate (3x100ml) and 100 ml water, dried over MgSO₄, and then evaporated. The resulting solid methoxysuccinyl 15 t-butyl glutamyl valine N-hydroxysuccinimide ester weighed 8.2 grams.

e) MeOSuc-Glu(t-Bu)-Val-Lys(Boc)

The MeOSuc-Glu(t-Bu)-Val-Osu (8.2g) prepared above was dissolved in 70 ml THF and added to a solution of 4.9g 20 epsilon-t-butyloxycarbonyl lysine in 60 ml water and 2.6 ml triethylamine. After stirring overnight the reaction mixture was diluted with 100 ml 2% sodium bicarbonate solution and extracted with ether. The aqueous solution was acidified with 3% citric acid and extracted with four 100 ml 25 portion of ethyl acetate. The combined organic layers were washed with 100 ml water, dried (MgSO₄) and evaporated to give 7.0 grams of hygroscopic solid methoxysuccinyl-gamma-t-butyloxycarbonyl lysine.

f) Fmoc-Glu(t-Bu)-Val-OBzl

30 This compound, as well as several in the next example, was prepared by a modification of the method of Anderson et al., J. Am. Chem. Soc. 89:5012 (1967).

- 29 -

A solution of 8.5 grams (20 millimoles) N-fluorenylmethoxy-carbonyl-gamma-t-butylglutamic acid in 80 ml anhydrous DMF was treated with 2.2 ml of NMM and cooled to -20°C under dry argon. A total of 2.6 ml isobutyl 5 chloroformate was then added dropwise. The solution was stirred for 10 minutes and a precooled (-20°C) mixture of 6.25g (25 millimoles) valine benzyl ester hydrochloride and 3.3 ml NMM in 50 ml DMF was added slowly. The reaction 10 mixture was stirred overnight and then dissolved in 300 ml ethyl acetate, and washed successively with water (1x100ml), 3% citric acid (3x100ml), dried (MgSO₄) and evaporated. The residue was dissolved in ethyl acetate and diluted with ether to give 11.8g of fluffy white crystalline 15 fluorenylmethoxycarbonyl-gamma-t-butyl-glutamyl-valine benzyl ester.

Example 2: Preparation of Peptide Substrates

a) Fmoc-Val-Lys(Boc)-Met-pNa

A solution of 1.1 grams of Fmoc-Val-Lys(Boc), 0.8 grams of BOP and 1.3 grams of 1-hydroxybenzotriazole (HOBr) 20 in 10 ml DMF was stirred for 10 minutes. A solution of 0.6g methionine p-nitroanilide and 1.5 ml acetic acid in 5 ml DMF and 0.3 ml NMM was then added. The reaction mixture was stirred overnight and diluted with 75 ml ethyl acetate. The solution was washed with water (2x30ml), 2% sodium bicarbonate (3x30ml), water (1x30ml), 3% citric acid (2x30ml), and water (1x30ml), and was dried (MgSO₄) and 25 evaporated under reduced pressure. The residue was dissolved in ethyl acetate and ether was added to precipitate 1.5g of a pale yellow solid 30 fluorenylmethoxycarbonyl-valyl-epsilon-t-butyloxycarbonyl-lysyl-methionine-p-nitroanilide.

b) Val-Lys(Boc)-Met-pNa

- 30 -

A solution of 1.5g Fmoc-Val-Lys(Boc)-Met-pNa in 20 ml methylene chloride was treated with 10 ml pyrrolidine. After 20 minutes the solvent was evaporated under reduced pressure. Remaining traces of pyrrolidine were removed by 5 repeated dilution with ethyl acetate followed by evaporation under reduced pressure. The resulting solid: valyl-epsilon-t-butyloxycarbonyl-lysyl-methionine-p-nitroanilide weighed 1.35g after washing with petroleum ether.

c) MeOSuc-Glu-Val-Lys-Met-pNa

10 A solution of 0.8g of MeOSuc-Glu(t-Bu) dicyclohexylamine salt, 1.3g of Val-Lys(Boc)-Met-pNa, 200mg of HOEt, 0.66g of BOP in 10 ml DMF was treated with 100 μ l of NMM. The reaction mixture was stirred for 3 days and then diluted with ethyl acetate and washed with water, 15 citric acid, and sodium bicarbonate and water as above. The organic layer was dried ($MgSO_4$) and evaporated to give a pale yellow solid which was purified by elution through a column prepared from 85g silica gel in chloroform. The first fraction, which was eluted with 150 ml chloroform, was 20 discarded. The product was next eluted with 300 ml of a solution of 2% methanol in chloroform. The resulting eluate was evaporated to give a colorless solid (0.45g) which was dissolved in 10 ml methylene chloride and 10 ml TFA. After 20 minutes the solvents were evaporated under reduced 25 pressure to give 270 mg of fluffy white solid methoxysuccinyl-glutamyl-valyl-lysyl-methionine-p-nitroanilide, which was purified by high pressure liquid chromatography (HPLC) in water using a gradient of 10 to 80% acetonitrile containing 0.1% TFA. The pure product weighed 30 80 milligrams.

d) MeOSuc-Glu-Val-Lys-Phe-pNa

- 31 -

This compound and the following two were prepared by a mixed carbonic anhydride procedure. A solution of 1.93g (3.0 millimoles) of MeOSuc-Glu(t-Bu)-Val-Lys(Boc) in 10 ml DMF and 0.33 ml NMM was cooled to -20°C, and 0.39 ml isobutyl chloroformate was added. The reaction mixture was stirred for 10 minutes at -20°C, and divided into three portions for this and the following two sections.

A 40% portion of the reaction solution containing 1.2 millimoles of the mixed isobutyl carbonic anhydride of 10 MeOSuc-Glu(t-Bu)-Val-Lys(Boc) was added under argon to a solution of 285 mg phenylalanine-p-nitroanilide (Bachem Biosciences, Philadelphia, PA.) in 5 ml DMF cooled to -15°C. After 30 minutes the reaction mixture was transferred to ice water and allowed to warm to room temperature over 2.5 hours. The reaction mixture was then diluted with 20 ml ether to precipitate a solid, which was filtered off, washed with ether and with petroleum ether, and dried. This solid was dissolved in 10 ml of a 1:1 mixture of methylene chloride and TFA. After 15 minutes, solvent was evaporated under reduced pressure, and the residue was triturated with ether to yield crude solid methoxysuccinyl-glutamyl-valyl-lysyl-phenylalanine-p-nitroan ilide. This was purified by HPLC in water containing 0.1% TFA using a 40 minute gradient of 25 to 90% acetonitrile containing 0.1% TFA.

e) MeOSuc-Glu-Val-Lys-D-Phe-pNa

This compound was made by the procedure given in section (d) above, except that D-Phenylalanine-nitroanilide was substituted for L-phenylalanine-p-nitroanilide.

f) MeOSuc-Glu-Val-Lys-Leu-pNa

This compound was made by the procedure given in section (d) above, except that leucine-p-nitroanilide was substituted for phenylalanine-p-nitroanilide.

- 32 -

The other protease substrates depicted in Fig. 11 were made by analogous methods.

Example 3: Preparation of Peptide Inhibitors

These compounds were prepared by the mixed carbonic anhydride procedure described in the previous example. In general, a completely protected precursor peptide carboxylate is converted to a mixed isobutylcarbonic anhydride and allowed to react at low temperature with the free amino group of the A1 residue which may optionally have its free functional groups protected as esters, ethers or acetals.

a) MeOSuc-Glu-Val-Lys-Phenylalanine
chloromethylketone
[N-Methoxysuccinyl-glutamyl-valyl-lysyl-(1-chloro-4-phenyl-2-keto-3-butylamide)].

The mixed isobutyl carbonic anhydride of methoxysuccinyl-gamma-t-butyl-glutamyl-valyl-epsilon-t-butoxycarbonyl lysine was prepared by dissolving 1.93g (3.0 millimoles) of this protected precursor peptide in 10 ml DMF and 0.33 ml NMM. The solution under dry argon was cooled to -20°C and 0.39 ml isobutyl chloroformate added. This reaction mixture was stirred for 10 minutes at -20°C, and divided into portions for this and the next two sections.

A 20% portion of the reaction solution (containing 0.6 millimoles of the precursor peptide mixed carbonic anhydride) was withdrawn and added to a solution of 142 mg (0.5 millimoles) of phenylalanine chloromethyl ketone hydrochloride (Bachem Biosciences, Philadelphia, PA.). The resulting solution was allowed to stir for three hours at room temperature, and then diluted with ether and filtered.

The filtrate was diluted with petroleum ether to precipitate MeOSuc-Glu-(t-Bu)-Val-Lys(Boc)-phenylalanine

- 33 -

chloromethyl ketone which was collected by filtration. This product was added to 10 ml of a 1:1 mixture of methylene chloride and TFA. After 15 minutes the solvents were evaporated under reduced pressure. The residue was

5 triturated with ether to give solid MeOSuc-Glu-Val-Lys-Phenylalanine chloromethylketone. This was purified by HPLC in 0.1% aqueous TFA on a C18 column with a 40 minute gradient of 25 to 90% acetonitrile.

b) MeOSuc-Glu-Val-Lys-Borophenylalanine pinacol

10 ester

A 20% portion of the mixed carbonic anhydride of MeOSuc-Glu(t-Bu)-Val-Lys(Boc) prepared in section (a) above was treated with 142 mg borophenylalanine pinacol ester (Shenvi, U.S. Patent 4,537,773; Shenvi and Kettner, J. Biol. Chem. 259, 15106, 1984), in 2 ml DMF chilled to -15°C. The solution was stirred for 30 minutes at -15°C, then placed in ice and allowed to warm to room temperature over 2.5 hours. The reaction mixture was diluted with ether and filtered. The filtrate was evaporated, and the residue dissolved in chloroform and loaded on a silica gel column (10g; E. Merck 20 Keiselgel 100). The product was eluted with 1:1 chloroform:methanol and fractions were monitored by thin layer chromatography (TLC).

The eluate was evaporated and the residue dissolved in 10 ml of 1:1 methylene chloride:TFA to remove the protecting groups. After 15 minutes solvent was evaporated and the residue triturated with ether. The resulting solid peptide product was purified by HPLC using conditions similar to those employed in section (a).

30 (c) MeOSuc-Glu-Val-Lys-Boronorleucine pinacol ester.

A 20% portion of the mixed carbonic anhydride of MeOSuc-Glu(t-Bu)-Val-Lys(Boc) prepared in section (a) above

- 34 -

was treated with 83 mg boronorleucine pinacolester (prepared by the method described in Shenvi and Kettner, *supra*), in 2 ml DMF. The reaction, workup and purification were carried out as described for the boro-Phe analog in (b) above.

5 (d) MeOSuc-Glu-Val-Lys-Norleucinal

A solution of 2.0 millimoles mixed carbonic anhydride was prepared from 1.29g MeOSuc-Glu(t-Bu)-Val-Lys(Boc), 7 ml DMF, 0.22 ml NMM and 0.25 ml isobutyl chloroformate by the procedure described in 10 section (a) above.

The resulting solution was added to a solution of 500 mg norleucinal di-n-butyl acetal (Gacek et al. Tetrahedron 30:4233, 1974) in 3 ml DMF. The reaction was carried out and worked up as described in section (b) above, 15 and the resulting product purified by HPLC.

(e) MeOSuc-Glu-Val-Lys-Phenylalaninal

This compound was prepared by the procedure of Example 3D except that phenylalaninal diethyl acetal was substituted for the norleucinal derivative, and a one hour 20 period of deprotection with 1:1 methylene chloride/TFA was employed. The product was purified by HPLC and the structure was verified by fast-atom bombardment mass spectroscopy (FAB-MS). Called M+1 = 620. Found M+1 = 620.

(f) MeOSuc-Glu-Val-Lys-Aza-Phe-NH₂

Potassium cyanate (0.73g, 0.009 mole) was added in 25 small portions over 30 minutes to a solution of 1.00g (0.0045 mole) of t-butyl 3-benzylcarbazate, and 4.5 ml of 4N hydrochloric acid in 5 ml of water and 5 ml of dioxane. The mixture was stirred overnight, and the white crystalline 30 product was filtered off and washed with aqueous 20% citric acid, water, and after vacuum drying weighed 0.81g. This solid was decrystallized from ethyl acetate-hexane to give

- 35 -

0.63g of N-t-Butoxycarbonyl-alpha-azaphenylalanine amide (Boc-AzaPhe-NH₂), m.p. 131-131.5°C (Lit. m.p. 124-125°C, Dutta et al., J. Chem. Soc. Perkin I (1975) 1712)

A solution of 0.6g of the above amide in 5 ml of dioxane and 5 ml of 4N HCl in dioxane was allowed to stand 1 hr, and the solvent was evaporated and 20 ml of ether was added. The precipitate (0.52g) was collected, washed with ether and dried. A 0.28g portion of the solid in 3 ml of DMF was treated with 1.2g of MeOSuc-Glu(t-Bu)-Val-Lys(Boc) (described in Example 1(e) above) and 0.33g of BOP and 0.11g of HOBT was added. The pH was adjusted by the dropwise addition of NMM (ca. 0.2ml) using a droplet of the reaction solution applied to moist pH paper to give a pH measurement of 7.5.. The solution was allowed to stand for 2 days and was diluted with 75 ml of ethyl acetate, and was worked up by the procedure in Example 1(c) above. The organic layer was evaporated to give 0.25g of a gummy solid which was taken up in 10 ml of 1:1 CH₂Cl₂/TFA. After 1 hr, 25 ml of ether was added to precipitate 0.15g of MeOSuc-Glu-Val-Lys-Aza-Phe-NH₂. The product was purified by HPLC and the identity was verified by FAB-MS.

Proteases

Proteases of this invention are chymotrypsin-like serine proteases derived from mammalian tissues. These proteases share an unusual substrate specificity in being able to cleave a Met-Asp bond in a beta-amyloid precursor protein. This aberrant cleavage is required to liberate beta-amyloid in Alzheimer's disease. The encompassed proteases have an enzymatic activity, as described above, which is similar to, or identical to, that activity naturally occurring in such mammals. These proteases may be isolated and purified, as generally described above, and

- 36 -

used as purified preparations. Alternatively, the amino acid sequence of the isolated protease, or tryptic or other fragments thereof, can be determined by standard techniques and the nucleic acid encoding that protease isolated. The 5 active site of the protease can be similarly determined by fragmenting the enzyme and testing its activity, e.g., as described below. Those fragments with the desired activity are useful in this invention. Fragments of the enzyme, like the intact enzyme, can also be created by standard 10 recombinant DNA technology. Antibodies, e.g., monoclonal antibodies, to these proteases or fragments thereof are created by standard technology.

Purified protease can be used for the rational design of organic molecules which fit into the enzyme active 15 site and inhibit its activity. The achievement of X-ray crystallographic structure of purified protease at high resolution will considerably aid this endeavor. Purified enzyme can also be used for large-scale screening of libraries of organic molecules to find compounds which 20 inhibit protease activity.

Examples of isolation of two such proteases, chymase and the multicatalytic protease, are described below. The examples are not limiting to the invention. Those of ordinary skill in the art can readily isolate other 25 proteases having such enzymatic activity using the protocol provided below, or equivalent protocols. Similarly, other proteases can be isolated by standard recombinant DNA technology using the amino acid sequence of chymase or the multicatalytic protease to allow reverse engineering of 30 nucleic acid encoding chymase, the multicatalytic protease, or related enzymes.

During the various protease purification steps, protease activity of all fractions was measured using

- 37 -

chromogenic or fluorogenic peptide analogs as substrates. Other methods are available, known to those skilled in the art. One specific example of an activity measurement is described here. Protease activity assays were performed in 5 96 well microtiter plates, in a total volume of 0.2 mls. Substrates were prepared as 10-20X concentrated stock solutions in DMSO. Therefore, each reaction contained 5-10% DMSO. Reactions were initiated by addition of 10 protease-containing samples to wells already containing substrates, and were maintained at 37°C. Control wells received either protease without substrate, or substrate 15 without protease. The release of reporter moieties by proteolytic cleavage was followed as a function of time by either spectrophotometry (e.g., for pNa substrates, change in absorbance with time at 405 nm) or fluorimetry (e.g., for AMC substrates, change in fluorescence with time using excitation at 390 nm and emission at 460 nm). Maximum velocity of reactions was determined while substrate hydrolysis increased linearly as a function of time.

20 Example 4: Chymase purification

Chymase (also known as mast cell protease I) was purified to homogeneity from rat brain generally as follows. Upon tissue homogenization and centrifugation, the enzyme was present in the particulate fraction. Chymase was 25 enriched by removal of detergent-soluble proteins. Chymase was then solubilized from the particulate fraction using high salt, such as 1M MgCl₂ or 2M NaCl. Next, it was precipitated by dialysis into a low ionic strength buffer, and was collected by centrifugation. The pellet was 30 suspended in buffer and fractionated by chromatography on a heparin-Sepharose column. Finally, chymase was purified to homogeneity by affinity chromatography using a substrate

- 38 -

linked to resin. Purification steps were monitored by hydrolysis of the general chymotrypsin-like protease substrate Suc-Ala-Ala-Pro-Phe-pNa (Nakajima et al., J. Biol. Chem. 254:4027, 1979), and the APP substrate mimics 5 MeOSuc-Glu-Val-Lys-Met-pNa and MeOSuc-Glu-Val-Lys-Phe-pNa. All procedures were performed at 4°C unless otherwise indicated.

a) tissue extractions

Forebrains from 5-7 day old rat pups (17g) were 10 homogenized using a glass/Teflon motorized homogenizer in 295 mls 20 mM sodium phosphate (pH 7.5), 1 mM EDTA, and 1% Triton X-100. After 30 minutes with occasional stirring, this homogenate was centrifuged for 1 hr. at 40,000 x g in an SS34 rotor. The pellets were resuspended in 40 ml 20 mM 15 sodium phosphate (pH 7.5), 1.2M MgCl₂, and 0.1% Triton X-100 using a large Dounce homogenizer. To obtain a tight pellet during the next centrifugation, the viscosity of the mixture was reduced by two passages through a 20-gauge needle to shear nucleic acids. After 40 minutes with occasional 20 stirring, the mixture was sonicated, then centrifuged as above for 1 hr. and the supernatant retained. This supernatant was then extensively dialyzed against a low 25 ionic strength buffer (5 mM sodium phosphate, pH 7.5). The following day, particulates present in the dialysis bag were first resuspended by end-to-end mixing, then the dialysate was centrifuged as above for 1 hr. The resulting pellet was dissolved in a total of 8 ml 20 mM sodium phosphate (pH 7.5), 1 mM EDTA, 0.1% Triton X-100 ("buffer A"), plus 0.4 M NaCl and was used directly for the heparin agarose column.

30 b) heparin affinity chromatography

The resuspension was sonicated, then applied in 1 ml aliquots to a column (1.5 x 2 cm) of heparin-agarose (Bio-Rad, Burlingame, CA.) equilibrated with buffer A plus

- 39 -

0.4 M NaCl. The column was washed with the same buffer until the concentration of eluting protein fell below 0.1 mg/ml. Proteins were then eluted from the column using a step-wise gradient of increasing NaCl concentration in 5 buffer A. The protease showed an avidity for heparin, and was eluted with 0.9-1.2 M NaCl, with an approximate enrichment of 20-fold. The above methods for partial purification of chymase (mast cell protease I) have been described in the literature (e.g., Woodbury et al., Meth. 10 Enzymol 80:588, 1981 and references therein).

c) Substrate affinity chromatography

The concentrated, partially purified chymase-containing sample was loaded onto a column containing Sepharose resin to which chymase substrate analog, Ala-Pro-Phe, had been covalently conjugated. The free amino group of the substrate analog was linked to Sepharose that had been activated by cyanogen bromide. Chymase bound to the resin in the presence of high concentrations of NaCl or MgCl₂, e.g., 1M NaCl, and was 15 eluted with 1M ammonium bicarbonate. Other methods are also available for chymase elution, e.g., reduction of NaCl to 20 0.5 M in 40% ethylene glycol. Eluted fractions were monitored for chymase activity as below.

d) Analyses of purified chymase

The eluate from the final column contained a closely-spaced doublet of ~25-26 kDa detectable by SDS-polyacrylamide gel electrophoresis with ultrasensitive silver staining (Nelson et al., J. Neurochem. 53:641, 1989; Merril et al., Anal. Biochem. 110:201, 1981). An 25 illustration is provided in Fig. 1. The silver-stained chymase doublet is shown in lane C. These polypeptides were 30 confirmed as proteases by enzymography. In this technique

- 40 -

(Miskin et al., Anal. Biochem. 118:252, 1981; Nelson and Siman, J. Neurochem., supra), protein samples that contain proteases are fractionated by SDS-polyacrylamide gel electrophoresis in which a protease substrate (e.g., 5 gelatin) is covalently incorporated into the gel matrix. Following electrophoresis, the SDS is removed and gels are incubated at elevated temperature (e.g., 37°C) to activate any proteases present. At locations within the gel containing active protease, the substrate will be cleaved from the gel matrix. The uncleaved substrate remaining 10 within the gel is stained by a protein-binding dye, such as Coomassie blue or Amido black. With this technique, proteases appear as light areas (where substrate has been liberated from the gel matrix) against a darkly stained 15 background. Purified chymase was examined by enzymography, shown in Figure 1 (Lane A is the heparin eluate, lane B the purified chymase; the left lane shows molecular weight standards, in kDa X 10⁻³). The two closely-spaced polypeptides in the purified chymase preparation (lane C) 20 both exhibit protease activity (lane B). The chymase activity exactly co-migrated with the protein detected by silver staining.

The purification scheme is summarized in Figure 2. Starting from 18g rat brain, approximately 10µg homogeneous 25 chymase was obtained. The purified protease exhibited characteristics of the chymotrypsin-like protease chymase: (1) molecular weight of ~25-26 kDa; (2) hydrolysis of peptide analog substrates with aromatic or large aliphatic amino acids at their carboxyl terminus (e.g., 30 Suc-Ala-Ala-Pro-Phe-pNa, MeOSuc-Ala-Ala-Pro-Met-pNa); (3) irreversible inhibition by and formation of an SDS-stable complex with alpha₁-antichymotrypsin.

- 41 -

Amino acid sequencing was performed on purified brain chymase to confirm the identity of the protease. This procedure was carried out by the Harvard Microchemistry Facility using a method modified from Aebersold et al.,
5 Proc. Natl. Acad. Sci. 84:6970 (1987). Chymase that had been eluted from the final substrate affinity column with ammonium bicarbonate was lyophilized. Next, ~150 pmol of chymase was digested with trypsin (enzyme:substrate of 1:20), and the resulting chymase fragments were separated by
10 narrow-bore reverse phase HPLC. Three fragments were chosen for automated N-terminal microsequencing on the basis of their yields and predicted lengths, and sequenced in a gas-phase sequenator. Figure 3 compares the partial amino acid sequences obtained from three fragments of the purified
15 protease with those published for authentic chymase (LeTrong et al., Biochemistry 26, 6988-6994, 1987). All 32 residues from the three domains precisely match the published sequence for chymase.

Example 5: Multicatalytic protease purification from human
20 brain

The multicatalytic protease identified in mammalian brain (Wilk and Orlowski, J. Neurochem. 40:842, 1983) has also been referred to in the literature as "ingensin" (Kamakura et al., J. Neursci. Res. 20:473, 1988), "lens
25 neutral endopeptidase" (Ray and Harris, Biochem. J. 248:643, 1987), "proteasome" (Tanaka et al., J. Biol. Chem. 261:15197, 1986); J. Biol. Chem. 263:16209, 1988), or "macropain" (Rivett, Arch. Biochem. Biophys. 268:1, 1989). We identified and purified from human brain an enzyme that
30 shares a number of features with the multicatalytic protease. The activity was present in the soluble fraction of a human brain homogenate, and bound to anion exchange and hydroxyapatite columns. Molecular weight of the protease

- 42 -

was found to be extremely large (>600,000) by gel filtration, a step that afforded significant purification of the enzyme. Protease activity at each stage of the purification was measured as described above. A specific example of multicatalytic protease purification from human brain is described below.

a) Tissue extraction and ion exchange chromatography
Human cerebral cortex (courtesy National Neurological Research Bank, Los Angeles, CA.) was obtained at autopsy and stored at -70°C until use. All procedures were conducted at 4°C unless noted otherwise. Three samples (35g) were pooled and homogenized in a Waring blender in 200 mls 10 mM sodium phosphate (pH 7.4), 0.5 mM EDTA. Triton X-100 was added to 0.5%, the homogenate was stirred for 1 hr., then centrifuged at 40,000 x g for 1 hr. The supernatant, containing the multicatalytic protease, was dialyzed against 20 mM Tris-HCl (pH 8.0), 1% glycerol. The following day, the dialysate was loaded on a column (150 ml bed volume) of DEAE-Sephadex Fast Flow (Pharmacia, Piscataway, N.J.) equilibrated in the dialysis buffer. Unbound material was removed with ~300 mls dialysis buffer, then bound material was eluted with a linear NaCl gradient from 0 mM to 400 mM (1.5 liters total). Fractions of 15 ml were collected and assayed for multicatalytic protease activity (see below). Figure 4 (top) shows the elution profile of the protease from this column. Protease activity was detected in this and subsequent purification steps by hydrolysis of the APP-mimic substrate MeOSuc-Glu-Val-Lys-Met-pNa (see example 6 below for details) in the presence of 0.05% SDS, which is known to stimulate multicatalytic protease activity (Ishiura et al., supra).

b) Gel filtration

- 43 -

Active fractions were pooled, brought to 30% saturated ammonium sulfate, stirred 30 minutes, and centrifuged at 40,000 \times g for 30 minutes. The pellet was discarded, while the supernatant was brought to 85% saturation and stirred and centrifuged as before. The 5 pellet, containing the multicatalytic protease, was suspended in 5 ml gel filtration running buffer (20 mM Tris-HCl, pH 7.5, 1% glycerol) and run on a 500 ml bed of Sephadryl S300HR (Pharmacia). The column was standardized 10 with molecular weight markers blue dextran (>2,000 kDa), thyroglobulin (670 kDa), gamma-globulin (150 kDa) and ovalbumin (43 kDa). The multicatalytic protease eluted from the column with an apparent molecular weight of approximately 700 kDa (Figure 4, middle).

15 c) Hydroxapatite chromatography

The human brain multicatalytic protease was further purified using hydroxyapatite-Ultrogel (IBF, Columbia, MD.); a 4 ml bed was equilibrated with 4 mM sodium phosphate (pH 7.4). Active fractions pooled from gel filtration were 20 diluted with equilibration buffer and loaded onto the column at room temperature. Unbound proteins were washed off with several column volumes of equilibration buffer, then bound proteins were eluted with a linear gradient of sodium phosphate of 4 mM to 400 mM (60 mls). The multicatalytic 25 protease eluted as illustrated in Figure 4 (bottom). This chromatographic step split the human brain protease activity into two peaks, as has previously been demonstrated for hydroxyapatite purification of the multicatalytic protease (Ishiura et al., supra).

30 Several features of the protease activity purified as above indicate that it is the human brain homolog of the multicatalytic protease: (1) size, ~700 kDa; (2) co-purification; (3) chymotrypsin-like activity, hydrolyzing

- 44 -

peptide analog substrates with aromatic or large aliphatic amino acids at the carboxy terminus; (4) marked stimulation of activity by low concentrations of SDS (Ishiura et al., supra); (5) inhibition by chymostatin, but not by several common protease blockers (e.g., phenylmethylsulfonylfluoride, leupeptin, pepstatin, o-phenanthroline, EDTA).

Example 6: Identification of proteases important to Alzheimer's disease.

Neuritic plaques are generated in the brain in Alzheimer's disease by aberrant liberation of beta-amyloid from its precursor proteins (APPs). The sequence of beta-amyloid contained within APPs is shown in Figure 5. Cleavage of the bond between Met⁵⁹⁶ (M in the one letter amino acid code) and Asp⁵⁹⁷ (D) is required for beta-amyloid formation, and has been proposed to occur by action of a chymotrypsin-like protease (Abraham and Potter, Biotechnology 7, 147-153, 1989); Van Nostrand et al., Nature 341, 546-549, 1989). Figure 5 illustrates a strategy employed by the inventors for identifying chymotrypsin-like proteases which can liberate beta-amyloid in Alzheimer's disease. Two novel protease substrates were designed and synthesized: MeOSuc-Glu-Val-Lys-Met-pNa and the 19 amino acid peptide referred to as "junction peptide". Because cleavage between -Glu-Val-Lys-Met and Asp- liberates beta-amyloid, proteases cleaving these substrates on the carboxy terminal side of Met could be involved in Alzheimer's disease.

Contrary to expectation, not all chymotrypsin-like proteases were able to cleave MeOSuc-Glu-Val-Lys-Met-pNa (Figure 6; EVKM using the one letter amino acid code). Both chymase and the multicatalytic protease readily degraded

- 45 -

this substrate, but the chymotrypsin-like enzymes cathepsin G and chymotrypsin were essentially without effect. This is particularly surprising, since both cathepsin G and chymotrypsin readily degraded another substrate with Met at the carboxyl terminus, MeOSuc-Ala-Ala-Pro-Met-pNa (e.g., Nakajima et al., supra). Selective cleavage of MeOSuc-Glu-Val-Lys-Met-pNa by chymase and the multicatalytic protease demonstrates that protease substrate specificity is markedly influenced by amino acid residues upstream of the cleavage site, and that not all chymotrypsin-like proteases are capable of liberating beta-amyloid. The results strongly implicate chymase and the multicatalytic protease in the abnormal production of neuritic plaque beta-amyloid.

Chymase was also tested for cleavage of a long peptide whose sequence spans the junction of the amino terminus of the beta-amyloid domain (residue Asp⁵⁹⁷). The 19 amino acid junction peptide, Glu-Val-Lys-Met-Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln (corresponding to APP residues 593 to 611, numbering according to Kang et al., supra), was synthesized by solid phase methods and purified by reverse phase HPLC. The structure was confirmed by FAB-mass spectrometry and automated microsequencing. Purified chymase was incubated with purified junction peptide at an enzyme:substrate molar ratio of 1:50 at room temperature. The reaction mixture was fractionated by reverse phase HPLC immediately after adding enzyme, as well as at 45 and 90 minutes and 24 and 48 hrs. The intact junction peptide alone was run on the same HPLC column to identify its retention time. In as little as 45 minutes, intact junction peptide disappeared and was replaced by three prominent peptide fragments (Figure 7). Each of these fragments was identified by automated

- 46 -

microsequencing. Based on the sequences of the junction peptide fragments, chymase degraded the junction peptide between Met and Asp. This further implicates chymase in the aberrant liberation of beta-amyloid in Alzheimer's disease.

5 The multicatalytic protease purified from human brain also exhibited an unusual ability to cleave the APP-mimic substrate MeOSuc-Glu-Val-Lys-Met-pNa (see example 8 below), like chymase but unlike the chymotrypsin-like enzymes chymotrypsin and cathepsin G. This implicates the
10 multicatalytic protease in the aberrant liberation of beta-amyloid in Alzheimer's disease.

Example 7: Human homolog of chymase

Human brain contains a homolog of rat chymase. The homolog was identified using the same two assays of chymase activity, hydrolysis of the chromogenic substrates Suc-Ala-Ala-Pro-Phe-pNa and MeOSuc-Glu-Val-Lys-Met-pNa, and enzymography, and was purified by a scheme similar to the one detailed above for the rat brain protease. The human brain chymase is similar to its rat counterpart in its co-purification, its inhibition by alpha₁-antichymotrypsin, its hydrolysis of peptide analog substrates of chymase, and its apparent molecular weight of about 22,000 on nonreducing enzymographic gels. Chymase was also detected in mast cells in human Alzheimer's disease brain using antibodies to authentic human mast cell chymase (provided by N. Schechter, University of Pennsylvania, described in Schechter et al., supra). Specific examples of these methods are described below.

Samples obtained post-mortem from human cerebral cortex (courtesy of the National Neurological Research Bank) were fractionated as described above for preparation of rat brain chymase. The human brain activity exhibited detergent-insolubility, high salt solubility, and low salt

- 47 -

insolubility. Protease was further purified on a heparin-Sepharose column in a manner similar to purification of rat brain chymase. Protease activity was monitored by hydrolysis of the substrate Suc-Ala-Ala-Pro-Phe-pNa as described above for rat chymase. The enzyme bound to the column in 0.4 M NaCl and was eluted by increasing the NaCl concentration to 0.7-0.9 M.

5 Next, chymase was purified using a chymase inhibitor coupled to resin, lima bean trypsin inhibitor-agarose (Sigma, St. Louis, MO.). This trypsin inhibitor binds to and blocks human mast cell chymase (Schechter et al., J. Biol. Chem. 258:2973, 1983). Chymase-containing fractions from the heparin eluate were pooled, concentrated to 10 ml, and loaded on a 1 ml bed volume of lima bean trypsin inhibitor-agarose. The column was washed with Hepes buffer containing 0.5 M NaCl (5 vol), then with water (5 vol). Chymase was eluted with 2 mM HCl, and was immediately neutralized with Tris-HCl (0.2 M final concentration, pH 8.0) containing 0.5 M NaCl. Figure 8 (right panel) summarizes the purification of the human brain protease. This enzyme hydrolyzed the general chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-pNa and also the specific substrate MeOSuc-Glu-Val-Lys-Met-pNa. The enzyme activity was inhibited by alpha₁-antichymotrypsin. On enzymographic gels, as shown in Figure 8, the human brain chymase exhibited protease activity which co-migrated with rat brain chymase (purified as described above) and authentic human mast cell chymase (Schechter et al., 1983, supra). Figures 9 and 10 illustrate the results of the purification.

20 30 Mast cell chymase was also located in human brain by immunohistochemical analysis using an antiserum to chymase described above. In this procedure, thin sections of human brain (obtained post-mortem, fixed in aldehydes, and

- 48 -

embedded in paraffin) are probed with rabbit antibody to human mast cell chymase. Bound rabbit anti-chymase is then detected by an indirect immunoperoxidase procedure (Sternberger, in Immunocytochemistry, Cohen et al. eds., 5 pp. 104-169, Wiley, New York, 1979); Siman et al., Neuron 3:279, 1989). Using this procedure, chymase immunoreactivity was located within the secretory granules of mast cells in both control and Alzheimer's disease cerebral cortex.

10. Example 8: Protease inhibitors

Inhibition of the proteases responsible for the aberrant liberation of beta-amyloid in Alzheimer's disease may slow the progression of the disease. Once the candidate proteases have been identified, on the basis of their ability to cleave the Met⁵⁹⁶-Asp⁵⁹⁷ bond in APP, the purified enzymes can be used to screen for protease inhibitors. Two examples of this approach are illustrated below, in the development of highly potent and selective inhibitors for the chymotrypsin-like proteases chymase and the 20 multicatalytic protease.

To develop inhibitors for the two proteases, over 40 peptide analog substrates were prepared by the methods described in examples 1 and 2 above. Additional substrates were purchased from Sigma or Bachem. These substrates 25 differ from one another by systematic, single residue substitutions; by comparing their cleavage by protease, the precise substrate preference of each protease was determined. In turn, this most-preferred sequence was used along with modifications of the carboxy terminal residue to 30 generate highly specific inhibitors. Those skilled in the art will recognize that a number of carboxy terminal modifications are available which convert a serine protease

- 49 -

substrate into an inhibitor (e.g., Powers et al., In Proteinase Inhibitors, Barrett et al. (eds) Elsevier, pp. 55-152, 1986). The inhibitors described below are not limiting to the invention; using equivalent approaches, 5 nonpeptidic molecules can be identified from screening collections of organic molecules, or can be rationally designed based on knowledge of the enzyme active site obtained from the series of peptide substrates.

a) Chymase inhibitors

10 Cleavage of 54 peptide analog p-nitroanilide substrates by brain chymase is depicted in Fig 11. At least 5 concentrations of each substrate were tested. Double-reciprocal plots were used to generate kinetic constants. In the figure, the V_{max}/K_m for each substrate is 15 presented compared with substrate #1, MeOSuc-Glu-Val-Lys-Phe-pNa. Residues upstream of the cleaved bond are denoted by their "P" position (P₁ is immediately upstream of the cleavage site, P₂ is two residues upstream, etc.).

20 Note that compounds 1-10 compare distinct P₁ amino acids. Among those examined, Phe was preferred by more than 8-fold over Nle, which in turn was preferred over Met and Leu. Other residues were not cleavable. Similar systematic comparisons were conducted for the P₂, P₃, and additional 25 upstream sites. Of particular note is the chymase preferences at the P₂ and P₄ positions: a positively charged side chain is preferred at P₂, while a negatively charged one is preferred at P₄. This substrate specificity distinguishes chymase from other members of the chymotrypsin 30 family. As already mentioned in example 6 above, neither chymotrypsin nor cathepsin G cleaved a substrate with negative charge at P₄ (Glu) and positive charge at P₂ (Lys), such as MeOSuc-Glu-Val-Lys-Met-pNa.

- 50 -

Based on the above chymase preferences, highly potent and selective inhibitors of the protease were prepared. By conversion of the carboxy terminal amino acid in a preferred substrate to an amino aldehyde, boronic acid, or chloromethylketone. Fig. 12 shows that the aldehyde, 5 boronic acid, and chloromethylketone analogs of MeOSuc-Glu-Val-Lys-Phe-pNa each inhibited chymase with a K_i of less than 20 nM. These are substantially more potent than any chymase inhibitor previously described in the literature. Moreover, they are highly selective for 10 chymase, as they inhibited other chymotrypsin-like proteases (Figure 12) or other proteases (Figure 11, panel A) only at much higher doses. For example, MeOSuc-Glu-Val-Lys-Phe-al, inhibits chymase with a K_i of 15 nM, but did not inhibit 15 chymotrypsin or cathepsin G at doses up to 100 times greater (Figure 12). Furthermore, the inhibitor caused substantial blockade of the proteases trypsin, thrombin, elastase, urokinase, plasmin, or plasma kallikrein only at 10 μ M concentrations or higher (Figure 11, panel A).

The aldehyde analogs of MeOSuc-Glu-Val-Lys-Phe and MeOSuc-Glu-Val-Lys-Nle were compared as chymase inhibitors. The Phe compound was approximately 20 times more potent than the Nle inhibitor, a finding consistent with the differential cleavage of the two p-nitroanilide substrate 25 versions of these compounds.

b) Multicatalytic protease inhibitors

A strategy equivalent to that used to design chymase inhibitors was employed for the human brain multicatalytic protease. Figure 14 shows the relative hydrolysis rates of 30 48 peptide analog substrates, compared at a single substrate concentration. Note that substrate preferences differ from those of brain chymase. For example, while chymase prefers Phe at P1 and Lys at P2, the multicatalytic protease prefers

- 51 -

Leu at P1 (although Phe, Nle, and Met are also excellent here) and Arg at P2. Among the residues tested thus far at the P3 position, Ile is the most-preferred. Thus, peptide analogs can be carefully tailored which target the 5 multicatalytic protease but spare related chymotrypsin-like enzymes.

As illustrated above for chymase, preferred substrates of the multicatalytic protease can be modified at their carboxy termini to generate potent enzyme inhibitors. 10 Figure 13, panel B depicts the inhibition of the multicatalytic protease by some peptide analogs and commercially available protease inhibitors. The novel peptide analog inhibitors are substantially more potent blockers of the multicatalytic protease chymotrypsin activity than any previously described inhibitor. 15

Example 9: AD and protease activity

Chymotrypsin-like protease activity was quantified in a number of human brain samples, in order to determine if chymase might be responsible for the aberrant proteolysis in 20 brain in AD. Ninety-five brain samples from four brain regions were obtained from the National Neurological Research Bank, Los Angeles, CA. Forty-seven were from patients diagnosed as having AD (diagnosis confirmed at time of death), and forty-eight were from non-demented 25 age-matched individuals who died of various causes. Chymase was enriched and separated from other protease activities by its insolubility in detergent and low-salt buffer, followed by solubilization in 1 M MgCl₂ (high salt extract preparation, as previously detailed). Chymase activity was 30 assessed by hydrolysis of the general chymotrypsin substrate MeOSuc-Ala-Ala-Pro-Phe-pNa and was normalized to total protein content.

- 52 -

In AD parietal cortex, chymase activity is significantly elevated by about 2-fold ($n= 17$; $p<0.05$; Figure 15). Activity is also elevated in frontal cortex ($n=24$), but is unchanged in occipital cortex ($n=26$) and 5 cerebellum ($n=28$). The differences in parietal and frontal cortex could not simply be ascribed to changes as a function of age or post-mortem sample autolysis time. Moreover, activity of two other proteases, cathepsin B and prolylendopeptidase, were not elevated in the same parietal 10 cortex samples, indicating that there is no generalized increase in protease activities in AD. It is noteworthy that the two brain areas exhibiting elevated chymotrypsin-like activity are severely afflicted by AD, whereas the two areas exhibiting no elevation of protease activity are relatively 15 spared by the disease (Brun et al., Histopathology 5 549, 1981); Kemper, in K. Nandy, ed., Senile Dementia: A Biomedical Approach, Elsevier, pp. 105-113, 1978). These results provides direct support for the idea that aberrant proteolytic activity of chymase might underlie the 20 pathogenesis of AD.

Therapy

The above inhibitors can be provided in any standard pharmaceutically acceptable buffer for administration to a patient suffering from AD or Down's Syndrome. Such 25 inhibitors can be administered by standard procedures preferably at a level between 10-1000 $\mu\text{g}/\text{kg}$, intravenously, intramuscularly, intranasally, orally or directly into brain tissue.

Other Embodiments

30 a) Nucleic acid encoding protease

Purified chymase or multicatalytic protease can be used for the cloning and sequencing of chymase and

- 53 -

multicatalytic protease cDNA and protein, by methods now familiar to those skilled in the art of molecular biology of proteins. Partial amino acid sequence can be obtained from purified human brain chymase using automated or manual peptide sequencing employing Edman degradation or other chemistries. The partial protein sequence can be used in two ways. In one, antibodies are raised against chymase. A synthetic peptide is prepared based on the partial sequence, and is conjugated to a carrier protein and used to raise polyclonal and monoclonal antibodies by conventional methods (Harlow et al., *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988). In the second, chymase cDNA is cloned and sequenced, thus providing the full nucleotide and amino acid sequences of the protein. An oligonucleotide is prepared based on the partial protein sequence, and is used to screen a cDNA library to select recombinant bacteria expressing chymase cDNA. Upon selection, the chymase cDNA is cloned and sequenced by conventional methods (Sambrook et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1989).

Alternatively, oligonucleotides based on the published amino acid sequence of rat chymase (LeTrong et al., *Biochemistry* 26:6988, 1987) can be used as primers for a polymerase chain reaction in the cloning of cDNA for rat or human chymase. Either the human cDNA can be directly obtained by using human mRNA to drive the reaction, or the rat cDNA can be obtained (by using rat mRNA to drive the reaction) and subsequently used as a probe for cloning the human counterpart by conventional methods.

Nucleic acid probes specific for chymase or the multicatalytic protease have utility in diagnostic formats using readily accessible cells. Mutations, duplication, or

- 54 -

overexpression of the genes for clipsin or the multicatalytic protease can be detected by a variety of methods known to those skilled in the art.

b) Antibodies to protease

Antibodies to chymase, the multicatalytic protease, or related proteases can be used in a variety of formats designed to detect protease aberrations which may be present in Alzheimer's disease. These have utility as diagnostic tests for the disease. For example, changes in the amount of chymase protein, or mutations in the chymase protein which lead to the production of altered forms, could be detectable in readily accessible cells such as skin fibroblasts or mast cells, or white blood cells. Antibody-based detection could be accomplished by a number of means presently in wide use by those skilled in the art, including Western blotting and enzyme-linked immunosorbant assay (ELISA; Harlow and Lane, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988).

c) Protease inhibitors

In addition to the inhibitors described in the examples, one skilled in the art may prepare other inhibitors of the enzymes of the invention using the above-described methods. A number of useful inhibitors are illustrated in Figure 16. The delineation of the enzymatic recognition requirements with the peptide substrates of the invention thus makes possible the design of inhibitors using similarly arranged peptide side chains. The removal of peptide bonds from the resulting molecules is, however, a desirable goal because the presence of such bonds may leave the inhibitor accessible to the attack of proteolytic enzymes. Additionally, such peptide bonds may affect the biological availability of the resulting therapeutic molecules. Several examples of the application of "depeptidization" to

- 55 -

the P3 and P4 positions (nomenclature of Berger and Schechter, Phil. Trans. R. Soc. London, Ser. B (1970) 257:249) are given in the list of equivalents in Fig. 16. In principle, this approach may be employed at the P1 and P2 5 positions by the substitution of the amide bond -CONH- with the spatially similar -CH₂CH₂-, -CH₂-O-, -CH=CH- and -CH₂S- groups.

- 56 -

Claims

1. A purified peptide having an endopeptidase enzymatic activity which causes hydrolysis of a peptide bond between a methionine and an aspartic acid in a beta-amyloid precursor protein.

2. A method for detection in a biological sample of a protease indicative of Alzheimer's disease, comprising the steps of:

(a) providing a substrate for said protease having the formula R-A4-A3-A2-A1-R1, wherein:
10 R is hydrogen or a N-terminal blocking group;
A4 is a covalent bond, an amino acid or a peptide;

15 R1 is a reporter group;
A3 is a covalent bond, a D-amino acid, Phe, Tyr, Val, or a conservative amino acid substituent of Val;
A2 is a hydrophobic amino acid, or Lys or conservative amino acid substituent of Lys, when A4 includes at least two amino acids, A2 is any amino acid; and
20 A1 is chosen from the group consisting of Met, Phe, Nle, Leu, Ile, Tyr, and 3-phenylproline;

(b) contacting said biological sample with said substrate; and
25 (c) detecting cleavage of said substrate as an indication of the presence of said protease in said biological sample.

3. The method of claim 2 wherein said biological sample is isolated from brain, liver, heart, muscle, white blood cells, mast cells, fibroblasts, serum, or
30 cerebrospinal fluid.

2077665

- 57 -

4. The method of claim 2 wherein said protease is chymase.

5. The method of claim 2 wherein said protease is multicatalytic protease.

6. A substrate for a protease, having the formula:
5 R-A4-A3-A2-A1-R1, wherein

R is hydrogen or a N-terminal blocking group;

A4 is a covalent bond, an amino acid or a peptide;

R1 is a reporter group;

10 A3 is a covalent bond, a D-amino acid, Phe, Tyr, Val, or a conservative amino acid substituent of Val;

A2 is a hydrophobic amino acid, or Lys or a conservative amino acid substituent of Lys or, when A4 includes at least two amino acids, A2 is any amino acid; and

15 A1 is chosen from the group consisting of Met, Phe, Nle, Leu, Ile, Tyr, and 3-phenylproline.

7. The substrate of claim 6, wherein A3 is chosen from the group consisting of Val, Nle, Nva, Ile, Leu, Tyr and Phe.

20 8. The substrate of claim 6, wherein A2 is chosen from the group consisting of Lys, Arg, Orn, Cit and Harg.

9. The substrate of claim 6, wherein A4 is chosen from Glu and Asp.

10. The substrate of claim 6, wherein R is chosen
25 from Boc, Fmoc, AC, Et, Suc, MeOSuc, Az, MeOAz, Ad, MeOAd, Sub, MeOSub, DNS, DNP, Z, or a D-amino acid.

- 58 -

11. The substrate of claim 6, wherein R₂ is chosen from pNa or AMC.

12. An inhibitor of a protease, having the formula R-A₄-A₃-A₂-Y, wherein

5 R is hydrogen, or a N-terminal blocking group;
A₄ is a covalent bond, an amino acid or a peptide;
A₃ is a covalent bond, a D-amino acid, Phe, Tyr,
Val, or a conservative amino acid substituent of Val;
A₂ is a hydrophobic amino acid or lysine or a
10 conservative amino acid substituent thereof or, when A₄
includes at least two amino acids, A₂ is any amino acid; and
Y is a group reactive with the active site of said
protease.

13. The inhibitor of claim 12, wherein Y has the
15 formula -HN-CH-R₃, wherein

|
R₂

R₂ is a lipophilic side chain substituent; and
R₃ is a functional group which reacts with a
20 hydroxyl group of said protease to form a transition-state
analog.

14. The inhibitor of claim 13 wherein said R₂ is a
saturated or unsaturated alkyl or substituted alkyl group
comprising two to ten carbon atoms or a alicyclic or
25 aromatic group comprising five to ten carbon atoms or a
arylalkyl group comprising eleven or fewer carbon atoms.

15. The inhibitor of claim 14 wherein said R₂ is an
ethyl, n-propyl, isopropyl, n-butyl, or sec-butyl group.

- 59 -

16. The inhibitor of claim 13 wherein said R3 group is -CHO, -COCH₃, COCH₂Cl, or COCH₂Br.

17. The inhibitor of claim 12 wherein said protease is chymase.

5 18. The inhibitor of claim 12 wherein said protease is multicatalytic protease.

19. A method for purifying a protease to at least 80% purity from a biological sample, comprising the steps of:

- 10 (a) homogenizing said biological sample in a low salt buffer including detergent;
- (b) isolating the particulate fraction from said homogenized biological sample;
- (c) dissolving said particulate fraction in a high
15 salt buffer;
- (d) isolating the soluble portion of said dissolved particulate fraction;
- (e) binding said protease to an ion exchange or a heparin column and then eluting said protease from said ion
20 exchange or heparin column; and
- (f) binding said protease to a substrate affinity column and then eluting said protease from said substrate affinity column.

20. The method of claim 19, wherein said biological
25 sample is isolated from a mammal.

- 60 -

21. The method of claim 20, wherein said mammal is chosen from the group consisting of human, rat, cow, and pig.

22. The method of claim 19, wherein said biological sample is isolated from brain, liver, heart, muscle, white blood cells, mast cells, or fibroblasts.

23. The method of claim 19, wherein said low salt comprises less than 0.5 M salt.

24. The method of claim 23, wherein said detergent is ionic or non-ionic.

25. The method of claim 24, wherein said detergent is Triton X-100.

26. The method of claim 19, wherein said particulate fraction is separated from the homogenized biological sample by centrifugation.

27. The method of claim 26, wherein said centrifugation is between 30,000 to 300,000 x g for 15 minutes to 120 minutes.

28. The method of claim 27, wherein said high salt is greater than 0.5 M salt.

29. The method of claim 19, wherein said soluble fraction is separated from the high salt by centrifugation.

- 61 -

30. The method of claim 29, wherein said centrifugation is at 30,000 to 300,000 x g for 15 minutes to 120 minutes.

31. The method of claim 19, wherein said protease 5 is bound to a heparin column.

32. The method of claim 19, wherein said affinity column comprises a substrate for said enzyme.

33. The method of claim 19, wherein said substrate has the formula: W-X-Y-Z, wherein
10 W is a covalent bond, an amino acid or a peptide;
X is valine or a conservative amino acid substituent thereof;
Y is a hydrophobic amino acid or Lys or a conservative substituent of Lys or, when W includes at least
15 two amino acids, Y is any amino acid; and
Z is chosen from the group consisting of Met, Phe,
Nle, Ile, Leu, Tyr, and 4-phenylproline.

34. The method of claim 19 wherein said protease is chymase.

20 35. The method of claim 19 wherein said protease is multicatalytic protease.

36. A method for treating a patient afflicted with Alzheimer's disease, said method comprising administering to said patient a protease inhibitor having the formula:
25 R-A4-A3-A2-Y, wherein
R is hydrogen or an N-terminal blocking group;
A4 is a covalent bond, an amino acid or a peptide;

- 62 -

A3 is a covalent bond, a D-amino acid, Phe, Tyr, Val, or a conservative amino acid substituent of Val;

A2 is a hydrophobic amino acid or Lys or a conservative amino acid substituent thereof or, when A4 includes at least two amino acids, A2 is any amino acid; and

Y is a group reactive with the active site of said protease.

37. The method of claim 36, wherein said administration is directly into the brain.

10 38. The method of claim 36, wherein said administration is intramuscular, oral, or intranasal.

39. A method for screening candidate chymase inhibitors comprising the steps of:

15 (a) incubating a chymase substrate with one or more said candidate chymase inhibitor and chymase; and

(b) determining whether said one or more candidate chymase inhibitors decrease the rate of cleavage of said substrate by said chymase.

40. A method for screening candidate multicatalytic 20 protease inhibitors comprising the steps of:

(a) incubating a multicatalytic protease substrate with one or more said candidate multicatalytic protease inhibitors and multicatalytic protease; and

25 (b) determining whether said one or more candidate multicatalytic protease inhibitors decrease the rate of cleavage of said substrate by said multicatalytic protease.

- 63 -

41. A method for screening candidate proteases capable of cleaving a beta-amyloid precursor protein between Met and Asp comprising the steps of

(a) providing a peptide having the sequence:

5 EVKMDAEFRHDSGYEVHHQ;

(b) contacting said candidate protease with said peptide; and

(c) determining whether said candidate protease cleaves said substrate.

10 42. A method for screening candidate proteases capable of cleaving a beta-amyloid precursor protein between Met and Asp comprising the steps of

(a) providing a substrate having the sequence:

R-Glu-Val-Lys-Met-R1, wherein

15 R is hydrogen or a N-terminal blocking group;

R1 is a reporter group;

(b) contacting said candidate protease with said substrate; and

(c) determining whether said candidate protease 20 cleaves said substrate.

43. A method for treating a patient afflicted with Alzheimer's disease, said method comprising administering to said patient an inhibitor of chymase in a pharmaceutically acceptable carrier.

25 44. A method for treating a patient afflicted with Alzheimer's disease, said method comprising administering to said patient an inhibitor of multicatalytic protease in a pharmaceutically acceptable carrier.

1/22

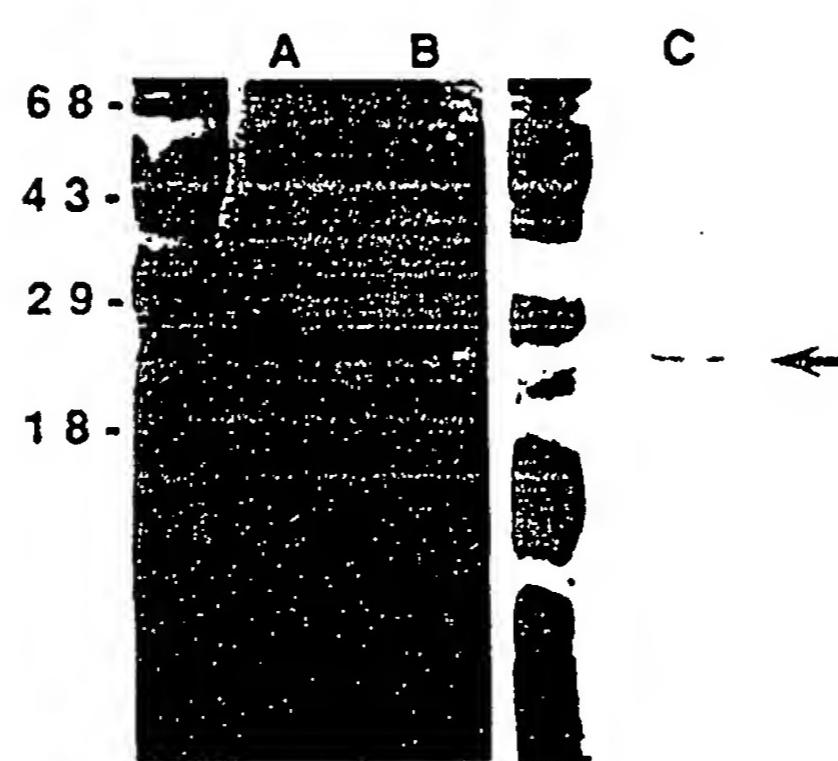


FIG. 1

SUBSTITUTE SHEET

2/22

FIG. 2*Purification of chymase from 7-day old rat brain*

Step	Volume ml	Protein mg	Units nmol / sec ^a	Specific activity units / mg	Yield %	Purification -fold
Homogenate	304	979	7,004	7	100	1
Detergent-extracted Pellet	40	180	5,043	28	72	4
Magnesium Extract	32	136	3,022	22	43	3
Low-Salt Precipitate	9	54	4,202	78	60	11
Heparin—agarose	8	1.8	2,677	1,487	38	212
Ala-Pro-Phe—Sephadex	2	0.01 ^b	2,387	238,700	34	34,100

^aOne unit of activity is defined as the release of one nanomole of p-nitroaniline per sec from AAPF-pNA at pH 7.5 at 37°C.

^bEstimated from amino acid analysis of the purified protein.

SUBSTITUTE SHEET

3/22

FIG. 3

Comparison between partial amino acid sequence of purified brain protease and rat mast cell protease I (RMCP I) sequence.

Brain IIGGVESRPHSRP[Y] A - L E I
RMCP I 1 IIGGVESRPHSRPYMAHLEI 21

Brain QIVHPNY
RMCP I 76 QIVHPNY 82

Brain ISPYVP[W]
RMCP I 211 ISPYVPW 219

SUBSTITUTE SHEET

4/22

Purification of human brain protease
homolog of the multicatalytic protease

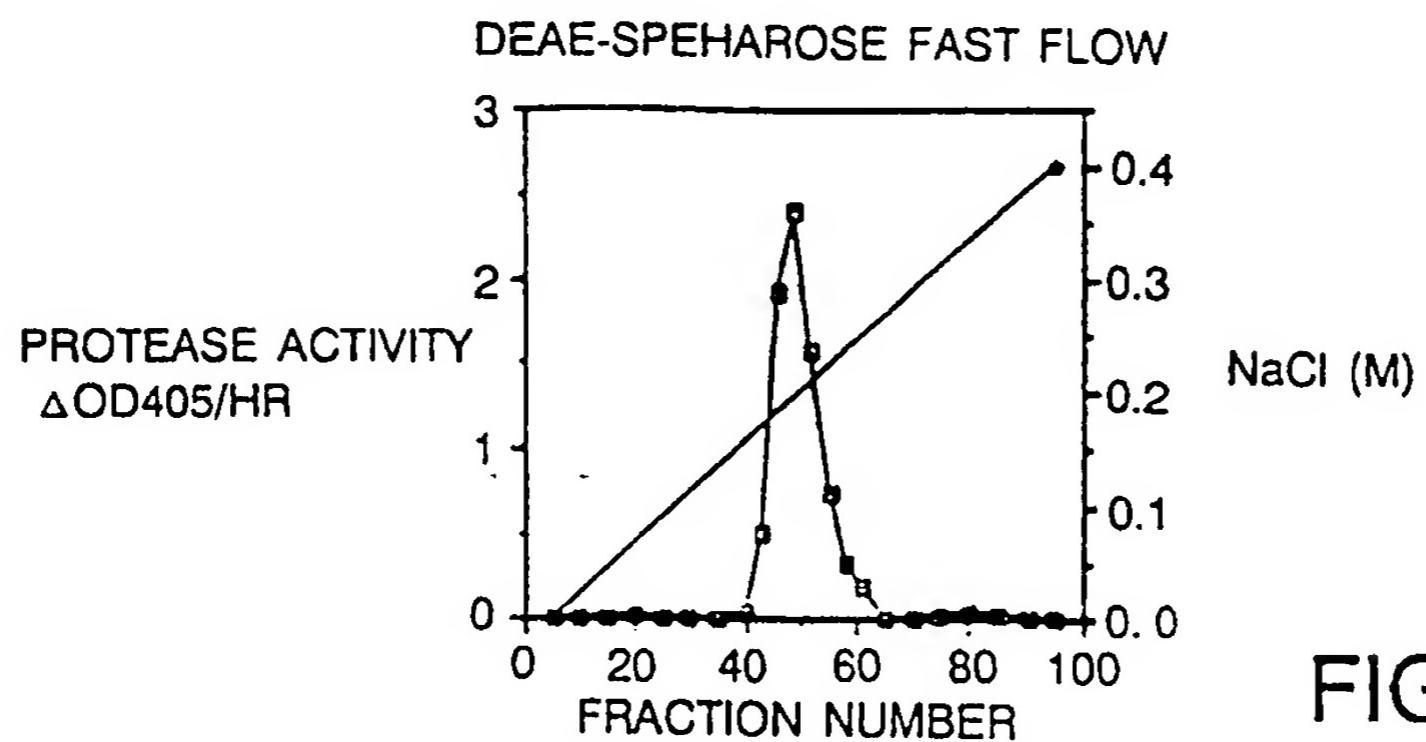


FIG. 4a

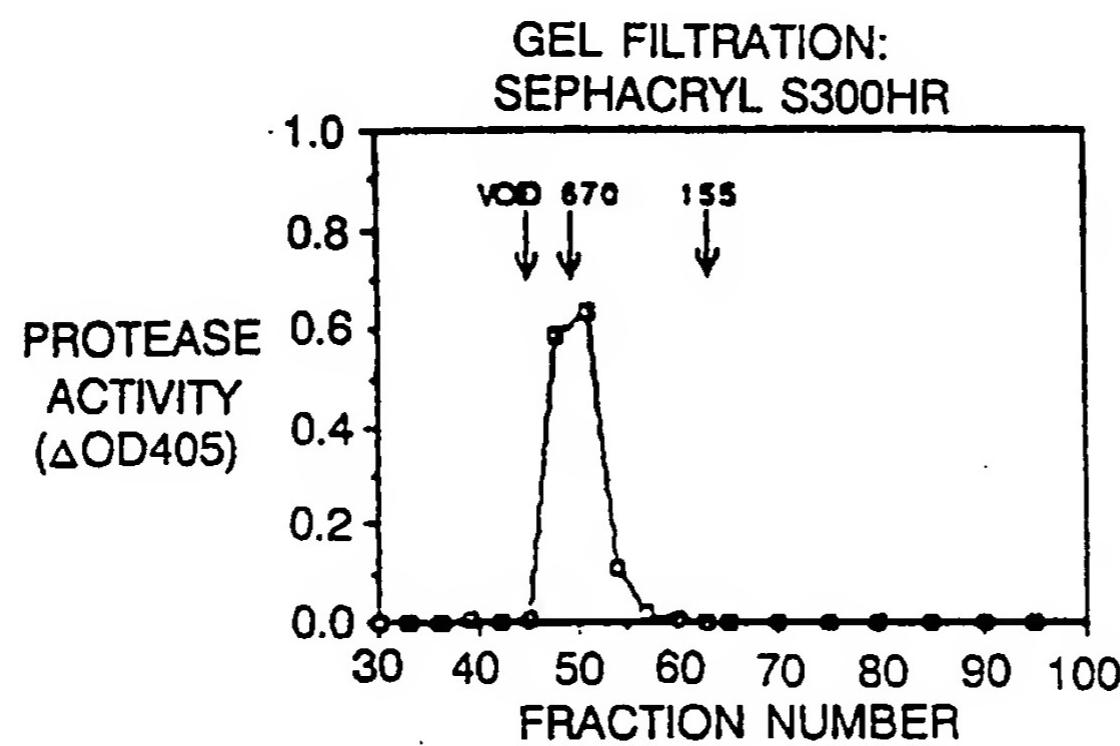


FIG. 4b

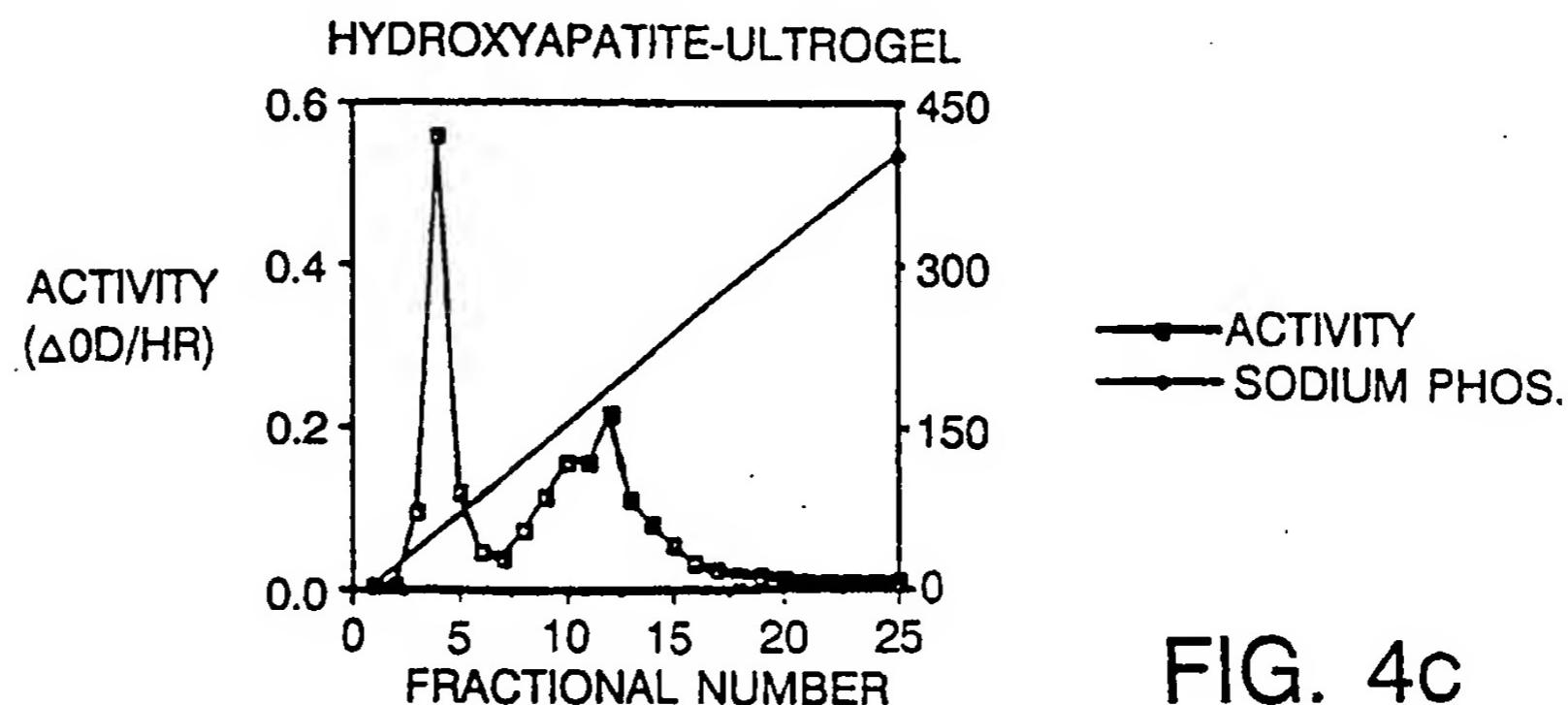
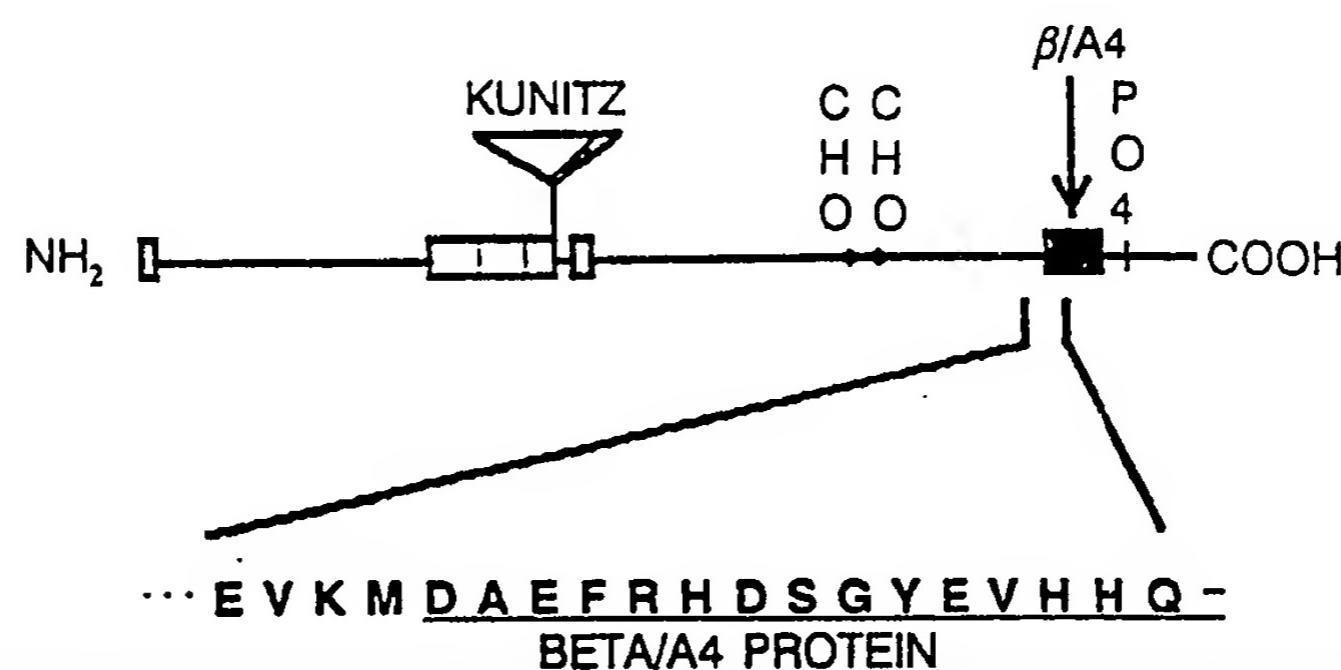


FIG. 4c

SUBSTITUTE SHEET

5/22

DESIGN OF PROTEASE SUBSTRATES FOR
IDENTIFICATION OF BRAIN PROTEASES
WHICH MAY GENERATE BETA/A4 PROTEIN



PROTEASE SUBSTRATES:

CHROMOGENIC: MeOSuc- EVKM -paranitroanilide

JUNCTION PEPTIDE: EVKMDAEFRHDSGYEVHHQ

FIG. 5

SUBSTITUTE SHEET

6/22

Brain chymase, but not cathepsin G or chymotrypsin, hydrolyzes the APP substrate mimic MeOSuc-Glu-Val-Lys-Met-pNa (EVKM) and related substrates

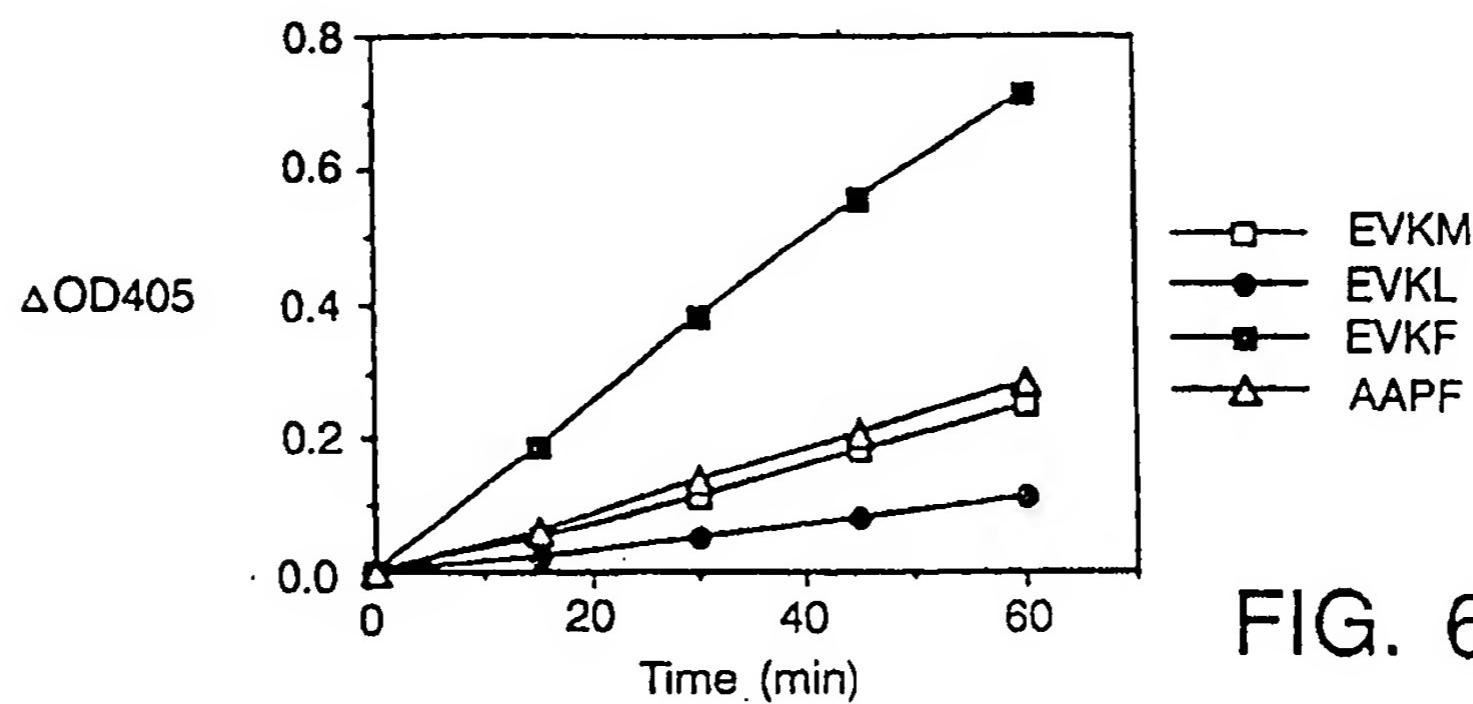


FIG. 6a

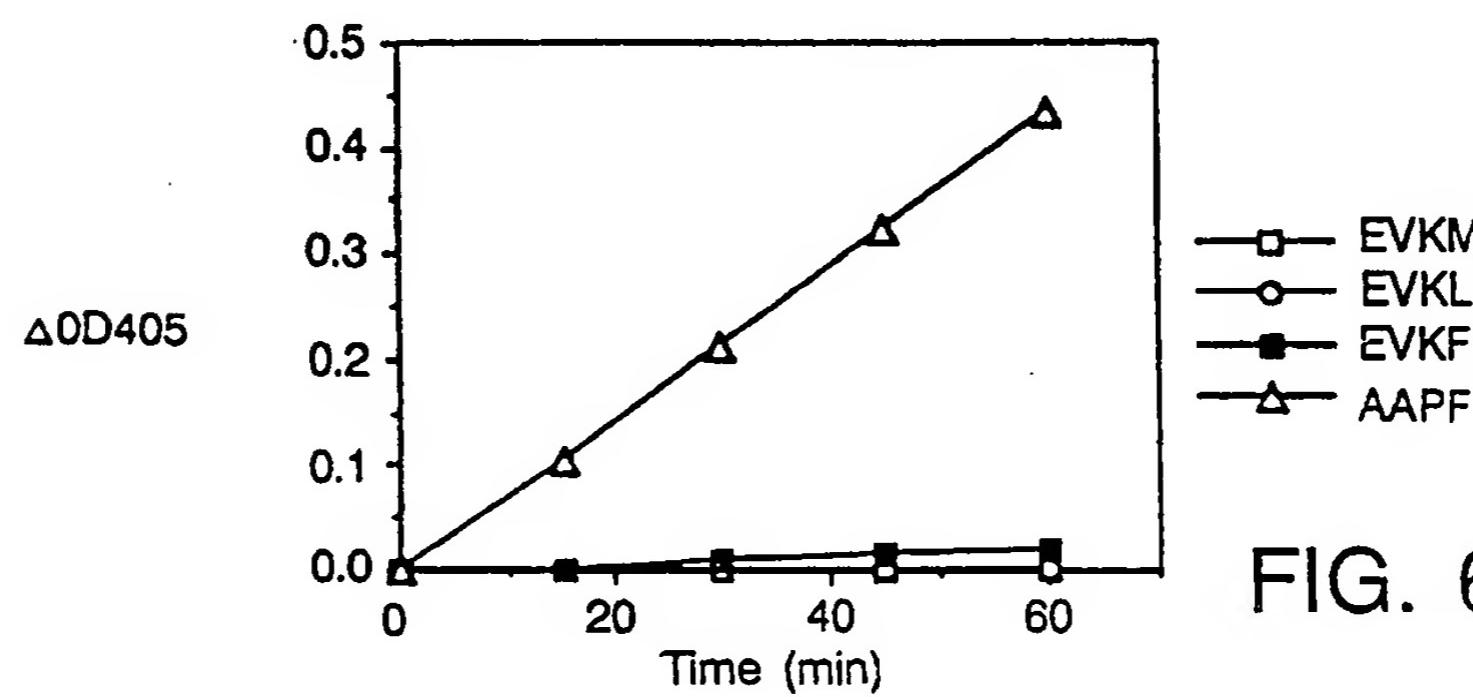


FIG. 6b

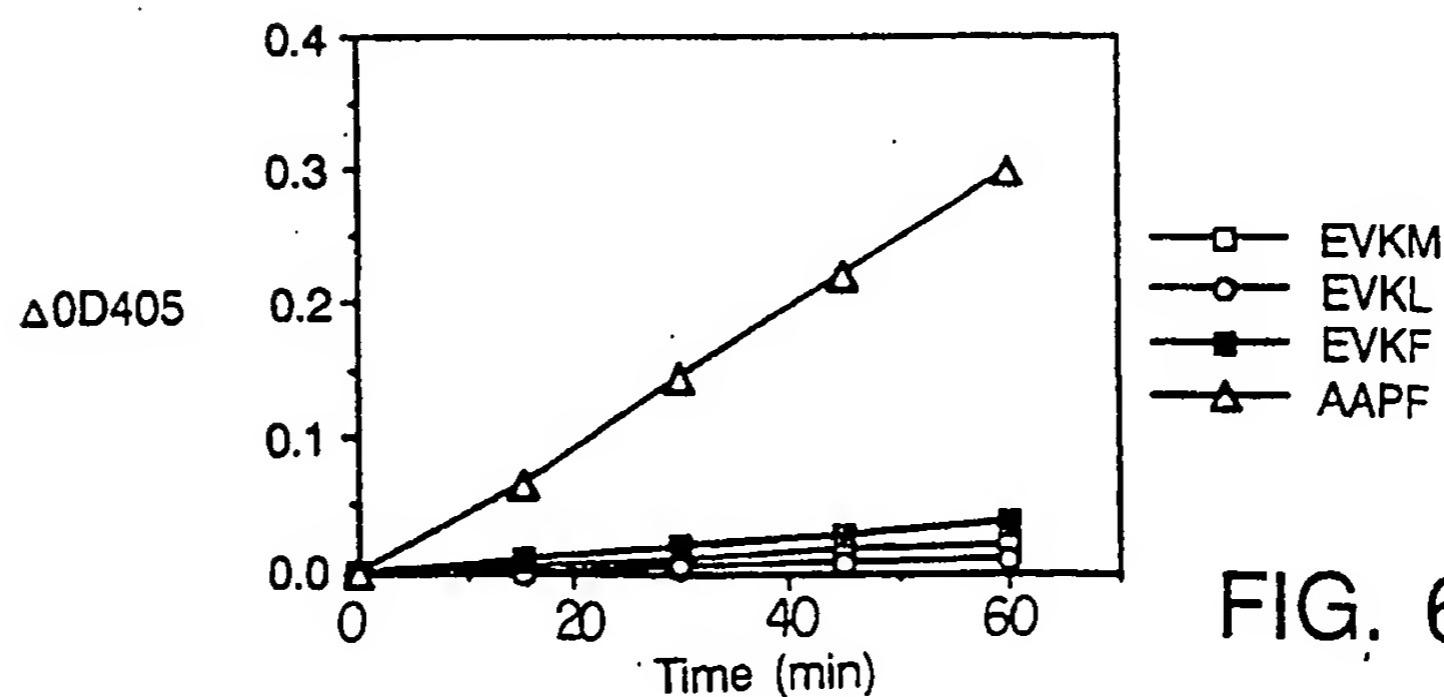
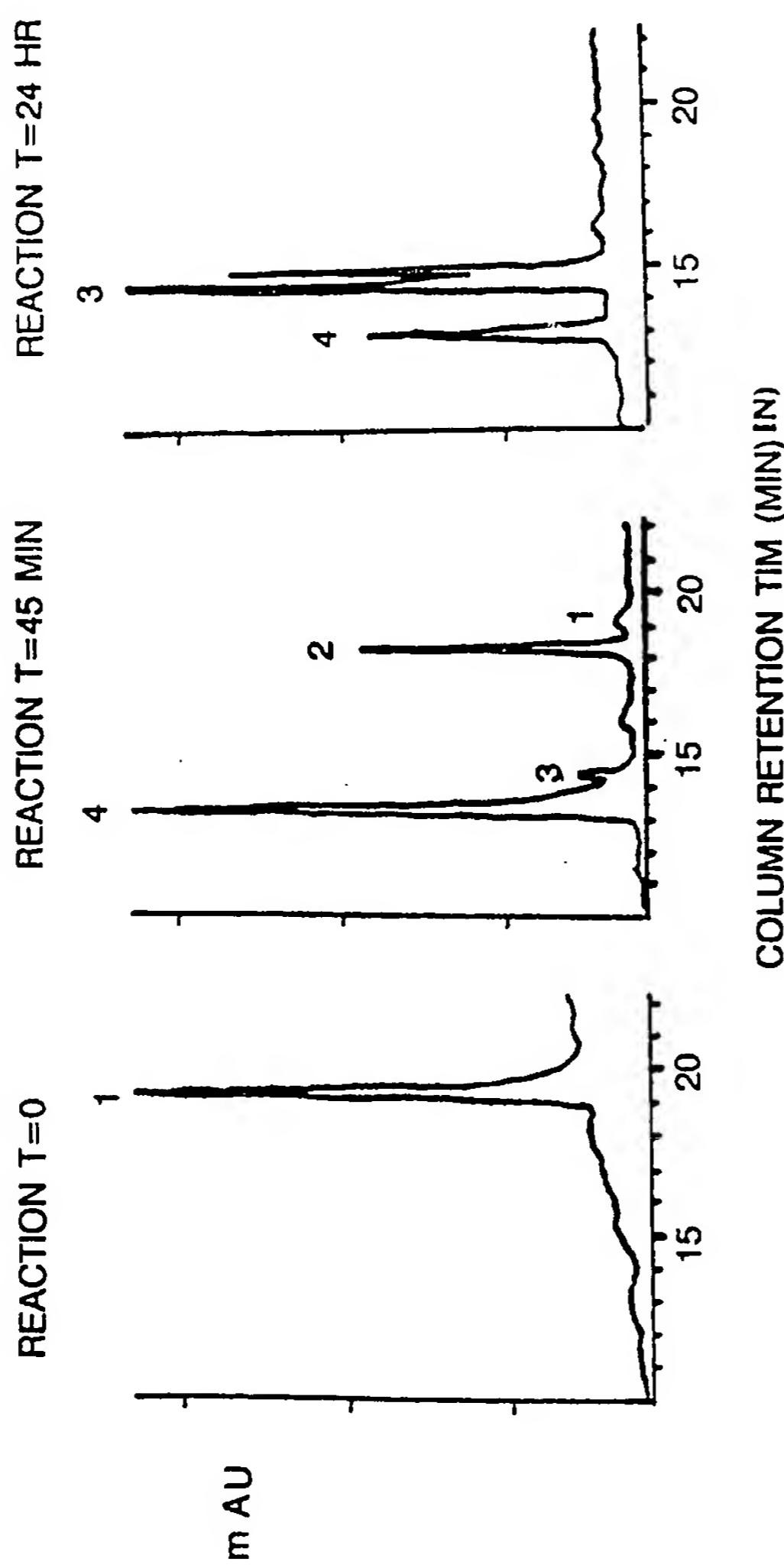


FIG. 6c

SUBSTITUTE SHEET

7/22



PEAK 1: EVKMDAEFRHDSGYEVHHQ
PEAK 2; EVKMDAEF

PEAK 3: DAEF

PEAK 4: RHDSGYEVHHQ

Chymase degrades an .APP-mimic junction peptide
between Met and Asp.

FIG. 7

SUBSTITUTE SHEET

8/22



FIG. 8

SUBSTITUTE SHEET

9/22

PURIFICATION STEP	CHYMO-LIKE ACTIVITY (U)	TOTAL PROTEIN (MG)	FOLD PURIFICATION
1. HOMOGENATE		9000	
2. DETERGEN-INSOLUBLE		4600	
3. WATER-INSOLUBLE		3750	
4. HIGH SALT EXTRACTION	5200	525	1
5. HEPARIN-SEPHAROSE	3100	6.0	52
6. TRYPSIN INHIBITOR-AGAROSE	765	0.070	1100

FIG. 9

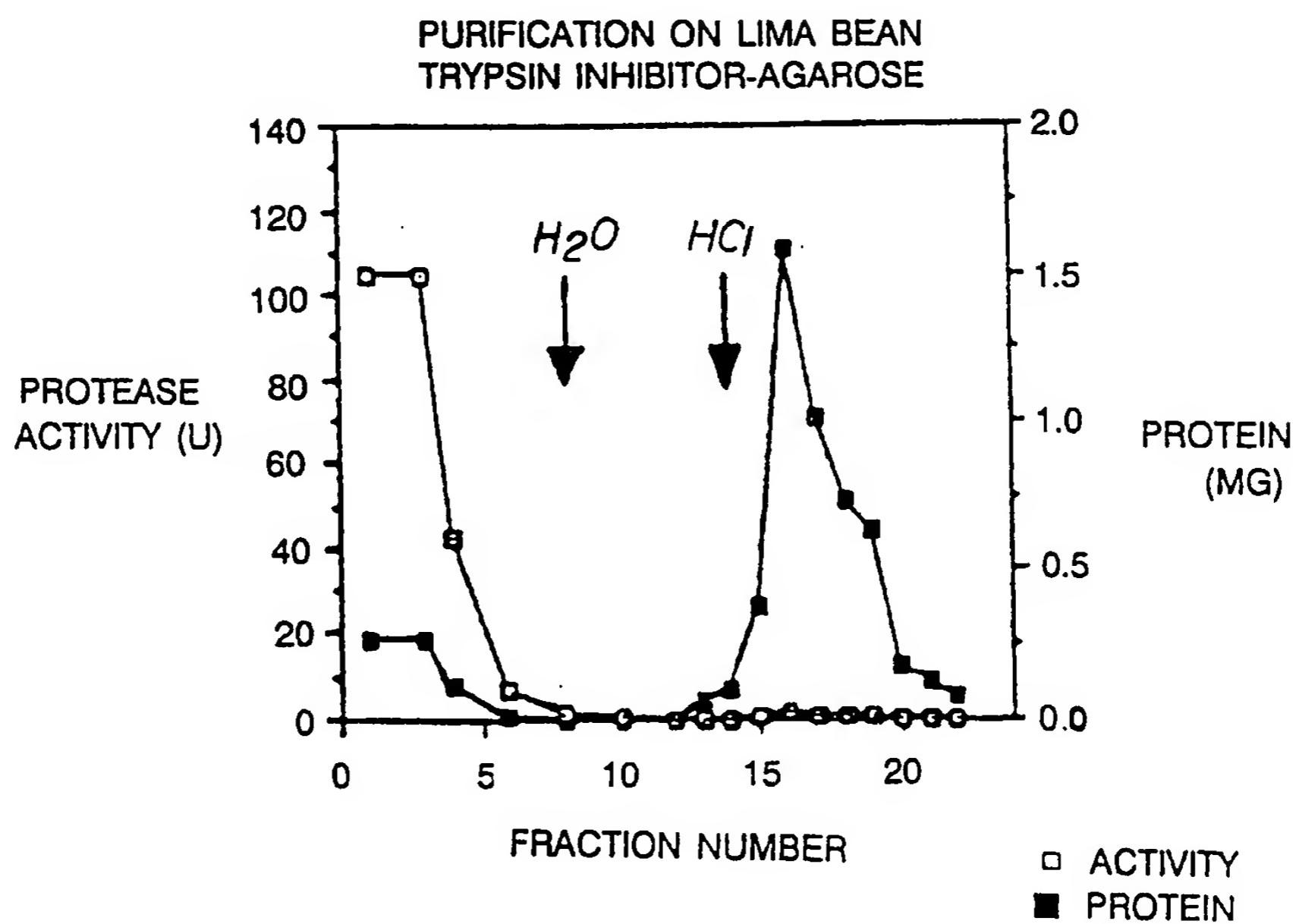


FIG. 10

SUBSTITUTE SHEET

10/22

FIG. 11 Rat brain chymase para-nitroanilide substrates

	P5	P4	P3	P2	P1	relative kcat/Km
1.	MeOSuc	Glu	Val	Lys	Phe	1000
2.	MeOSuc	Glu	Val	Lys	Met	40
3.	MeOSuc	Glu	Val	Lys	Leu	41
4.	MeOSuc	Glu	Val	Lys	d-Phe	<1
5.	MeOSuc	Glu	Val	Lys	Nle	124
6.	Suc	Ala	Ala	Pro	Phe	69
7.	Suc	Ala	Ala	Pro	Met	6
8.	Suc	Ala	Ala	Pro	Val	<1
9.	Suc	Ala	Ala	Pro	Ala	<1
10.		MeOSuc	Glu	Val	Lys	<1
11.		Sub	Val	Lys	Phe	1304
12.		MeOSub	Val	Lys	Phe	959
13.		Az	Val	Lys	Phe	1235
14.		MAz	Val	Lys	Phe	377
15.		MAd	Val	Lys	Phe	1166
16.		Ad	Val	Lys	Phe	2213
17.		d-Glu	Val	Lys	Phe	546
18.		Isobutyryl	Glu	Leu	Phe	230
19.		Ac	Glu	Leu	Phe	240
20.	I-Ser	Glu	Val	Lys	Phe	747
21.	d-Ser	Glu	Val	Lys	Phe	848
22.	Boc-d-Ser	Glu	Val	Lys	Phe	855
23.	MeOSuc-Ser	Glu	Val	Lys	Phe	908
24.	MeOSuc-Ile-Ser	Glu	Val	Lys	Phe	1361
25.	Ac	Glu	Val	Lys	Phe	573
26.	Biotin	Glu	Val	Lys	Phe	158
27.	Z	Glu	Val	Lys	Phe	887
28.	Ac	Ala	Ala	Pro	Phe	41
29.		Suc	Ala	Ala	Phe	56
30.			Ala	Ala	Phe	32
31.		Suc	Phe	Leu	Phe	434
32.		Suc	Val	Lys	Phe	1060
33.		Suc	Gly	Gly	Phe	8
34.		MeOSuc	Val	Lys	Phe	414
35.		MeOSuc	Glu	Lys	Phe	255
36.	MeOSuc	Glu	Phe	Lys	Phe	361
37.	MeOSuc	Glu	Phe	Glu	Phe	21
38.	MeOSuc	Glu	Val	Orn	Phe	586
39.	MeOSuc	Glu	Val	Arg	Phe	622
40.	MeOSuc	Glu	Val	Nle	Phe	300
41.	MeOSuc	Glu	Val	Phe	Phe	214
42.	MeOSuc	Glu	Val	Pro	Phe	493
43.	MeOSuc	Glu	Val	Leu	Phe	1144
44.	MeOSuc	Asp	Val	Lys	Phe	947
45.	MeOSuc	Val	Glu	Lys	Phe	447
46.	MeOSuc	Lys	Glu	Lys	Phe	328
47.	MeOSuc	Glu	Ala	Lys	Phe	551
48.	MeOSuc	Glu	Gly	Lys	Phe	14
49.	MeOSuc	Glu	Aib	Lys	Phe	<10
50.	MeOSuc	Glu	Nle	Lys	Phe	658
51.	Suc	Ala	Lys	Pro	Phe	26
52.	Suc	Ala	Gln	Pro	Phe	201
53.	Suc	Ala	Glu	Pro	Phe	217
54.	Suc	Ala	Phe	Pro	Phe	190

SUBSTITUTE SHEET

11/22

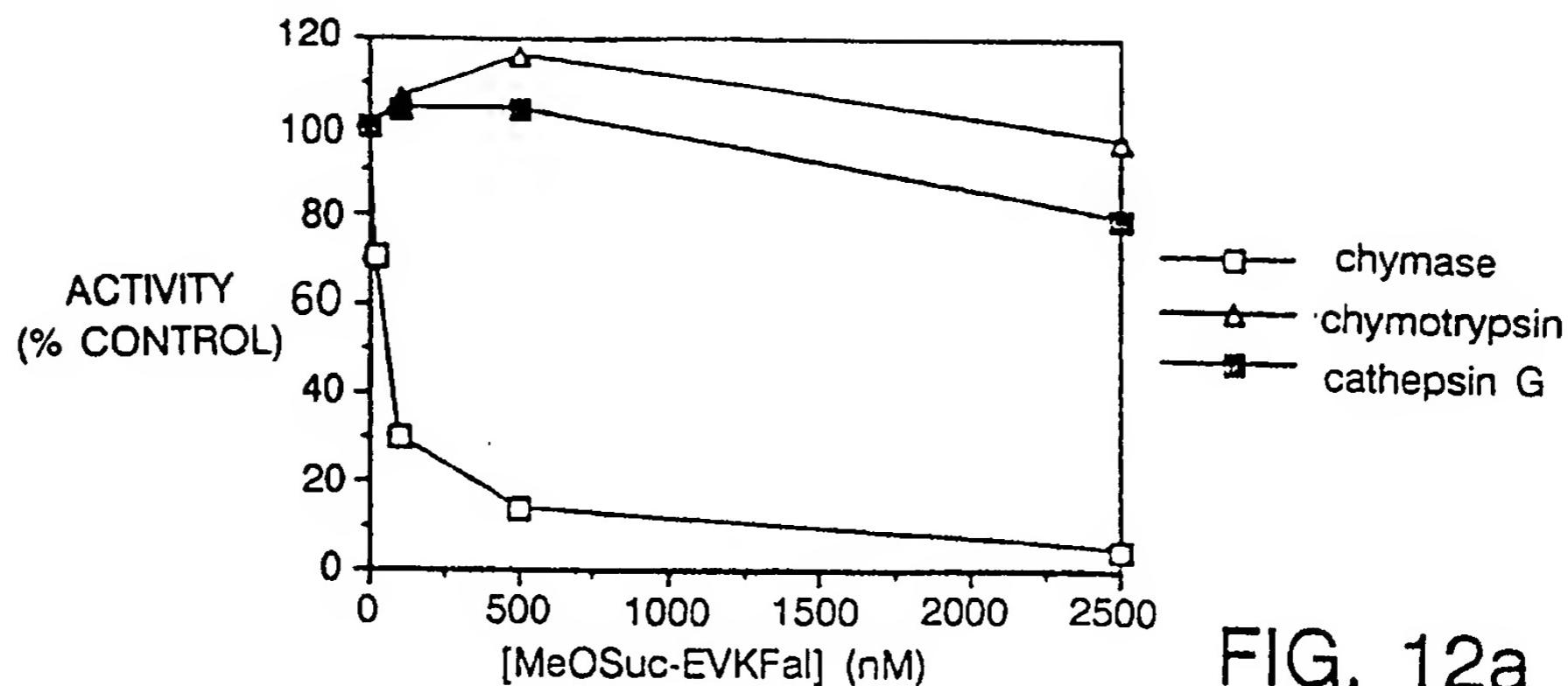


FIG. 12a

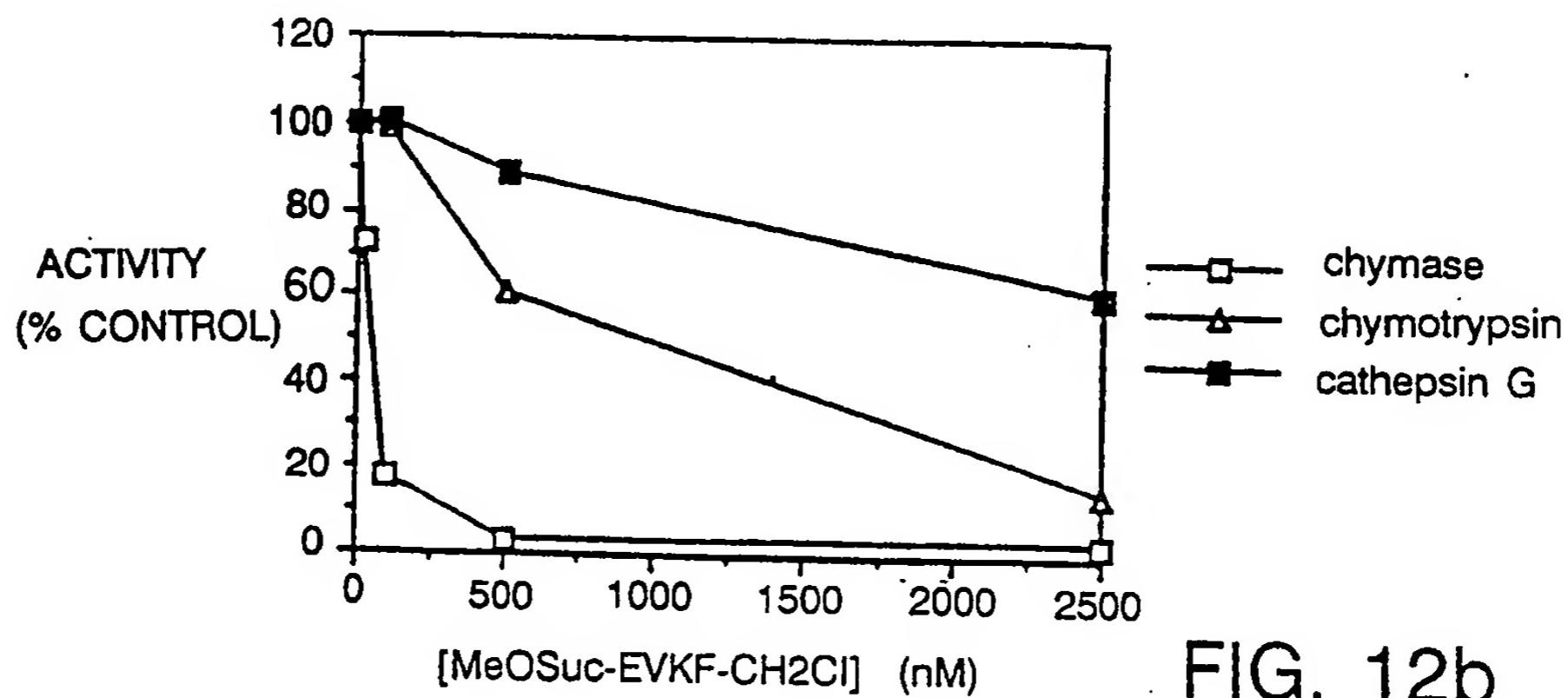


FIG. 12b

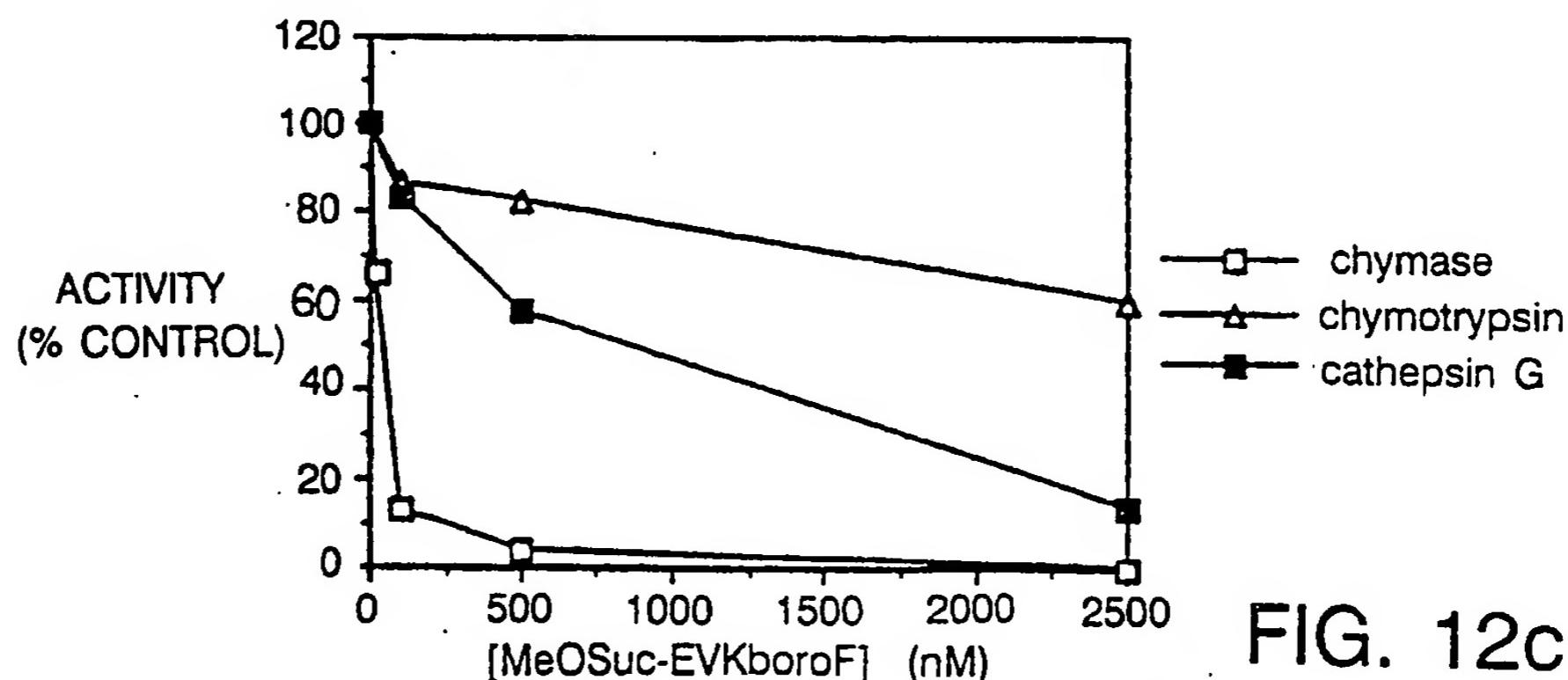


FIG. 12c

SUBSTITUTE SHEET

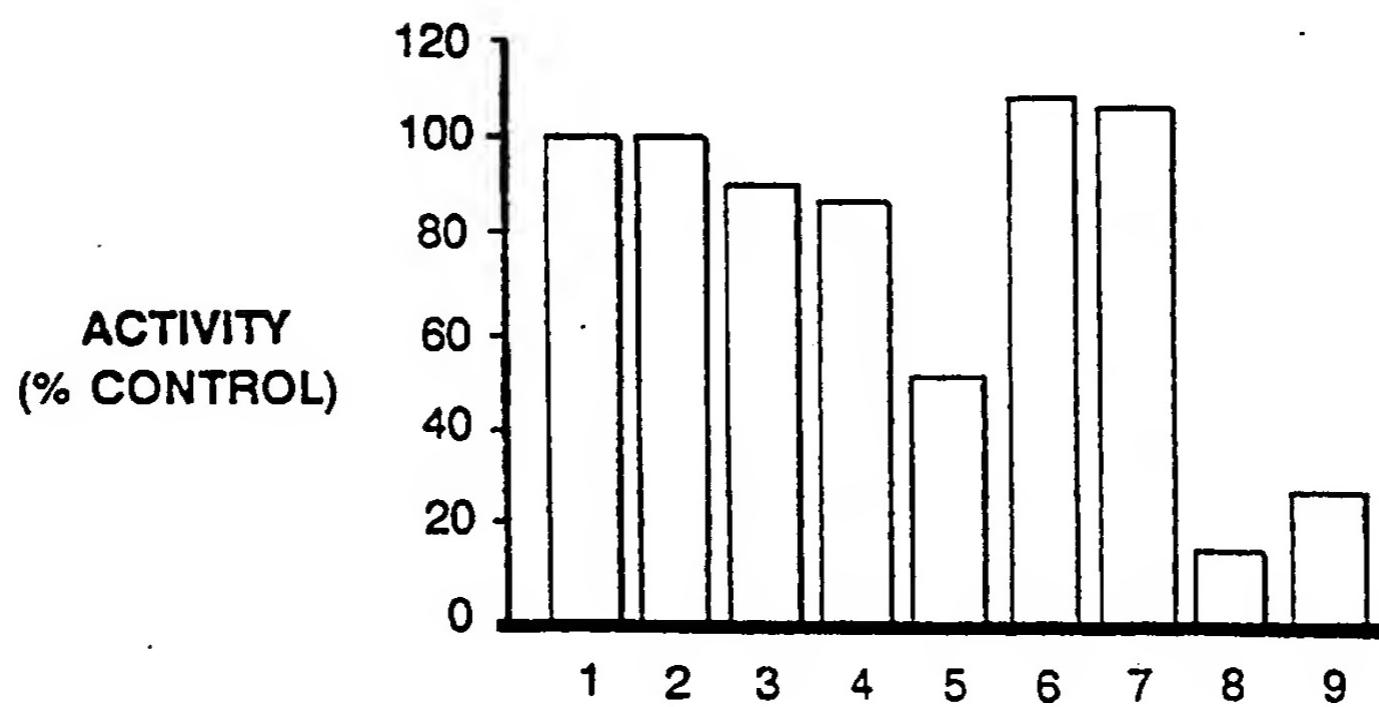
12/22

Inhibition of various proteases
by MeOSuc-Glu-Val-Lys-Phe-aldehyde

<u>protease</u>	<u>%inhibition at 10 μM</u>
chymase, rat brain	Ki=15 nM
multicatalytic protease, human brain	92
elastase, porcine pancreas	0
thrombin, human plasma	0
plasmin, human plasma	80
cathepsin B, bovine spleen	83
urokinase, human kidney	15
trypsin, bovine pancreas	20
kallikrein, human plasma	0

FIG. 13a

INHIBITION OF MULTICATALYTIC PROTEASE



- 1 NO INHIBITOR
- 2 ANTICHY MOTRYP SIN, 5 μ g/ml
- 3 PHENYL METHYL SULFONYL FLUORIDE, 100 μ M
- 4 LEUPEPTIN, 50 μ M
- 5 N-ETHYL AMALEIMIDE, 1 mM
- 6 PEPSTATIN, 10 μ M
- 7 EDTA, 1 mM
- 8 MeOSuc-EYKFal, 10 μ M
- 9 MeOSuc-EYKboroF, 10 μ M

FIG. 13b

SUBSTITUTE SHEET

13/22

FIG. 14

Multicatalytic Protease Activity: % relative hydrolysis

	P5	P4	P3	P2	P1	Hydrolysis
1.	MeOSuc	Glu	Val	Lys	Met	100
2.	MeOSuc	Glu	Val	Lys	Phe	104
3.	MeOSuc	Glu	Val	Lys	d-Phe	0
4.	MeOSuc	Glu	Val	Lys	Nle	110
5.	MeOSuc	Glu	Val	Lys	Leu	158
6.		MeOSuc	Glu	Val	Lys	0
7.	MeOSuc	Glu	Val	Pro	Phe	0
8.	MeOSuc	Glu	Val	Arg	Phe	432
9.	MeOSuc	Glu	Val	Orn	Phe	163
10.	MeOSuc	Glu	Val	Glu	Phe	0
11.	MeOSuc	Glu	Val	Phe	Phe	137
12.	MeOSuc	Glu	Val	Leu	Phe	70
13.	MeOSuc	Glu	Val	Nle	Phe	82
14.	Suc	Ala	Ala	Pro	Phe	0
15.	Suc	Ala	Ala	Pro	Met	0
16.		Suc	Gly	Gly	Phe	2
17.	MeOSuc	Glu	Phe	Lys	Phe	162
18.	MeOSuc	Glu	Ala	Lys	Phe	51
19.	MeOSuc	Glu	Nle	Lys	Phe	66
20.	MeOSuc	Glu	Gly	Lys	Phe	5
21.	MeOSuc	Lys	Val	Lys	Phe	32
22.	MeOSuc	Val	Glu	Lys	Phe	125
23.		MeOSuc	Ile	Lys	Phe	272
24.		MeOSuc	Gly	Lys	Phe	29
25.		Isobutyryl	d-Glu	Lys	Phe	0
26.			d-Glu	Lys	Phe	0
27.	Suc	Glu	Aib	Lys	Phe	0
28.		MeOSuc	Ala	Lys	Phe	27
29.		MeOSuc	Pro	Lys	Phe	10
30.		MeOSuc	d-Pro	Lys	Phe	0
31.			Ad	Lys	Phe	20
32.			Az	Lys	Phe	38
33.		MeOSuc	Val	Lys	Phe	155
34.		Suc	Val	Lys	Phe	80
35.		MeOAZ	Val	Lys	Phe	81
36.		Az	Val	Lys	Phe	54
37.		MeOSub	Val	Lys	Phe	127
38.		Sub	Val	Lys	Phe	107
39.		MeOAd	Val	Lys	Phe	207
40.		Ad	Val	Lys	Phe	228
41.		Z	Val	Lys	Phe	82
42.		Boc	Ala	Ala	Phe	69
43.		Suc	Ala	Ala	Phe	11
44.			Ala	Ala	Phe	14
45.	Z	Glu	Val	Lys	Phe	478
46.	d-Ser	Glu	Val	Lys	Phe	64
47.	Biotin	Glu	Val	Lys	Phe	10
48.	Ac	Glu	Val	Lys	Phe	66

SUBSTITUTE SHEET

2077665

14/22

CHYMOTRYPSIN-LIKE ACTIVITY IN AGED HUMAN BRAIN

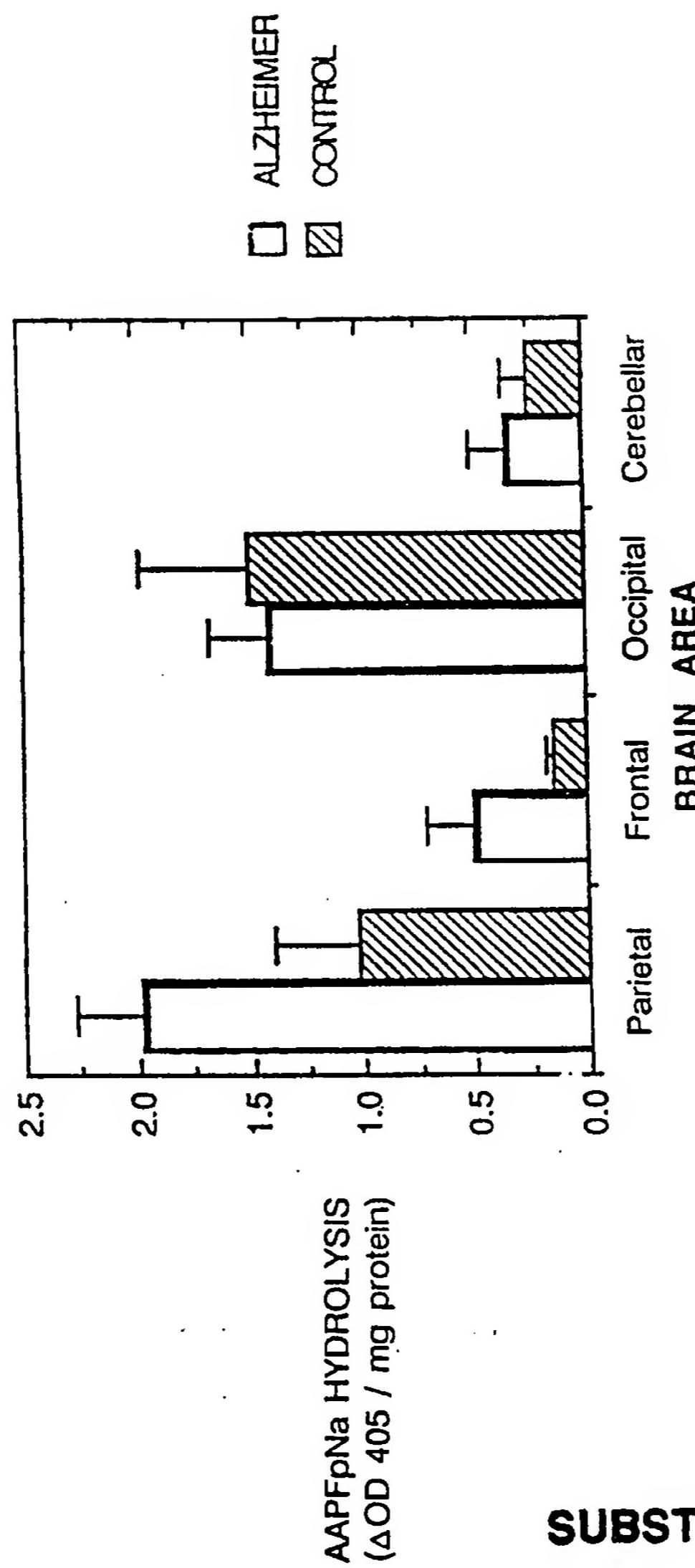


FIG. 15

SUBSTITUTE SHEET

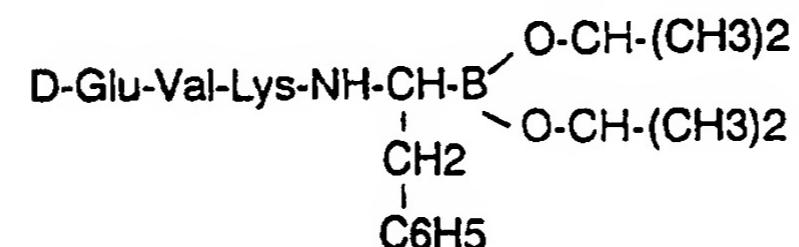
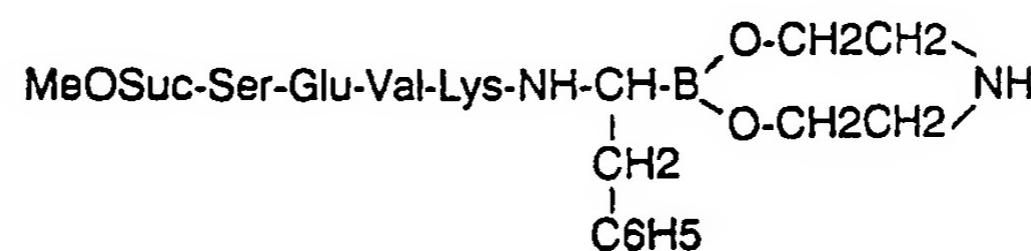
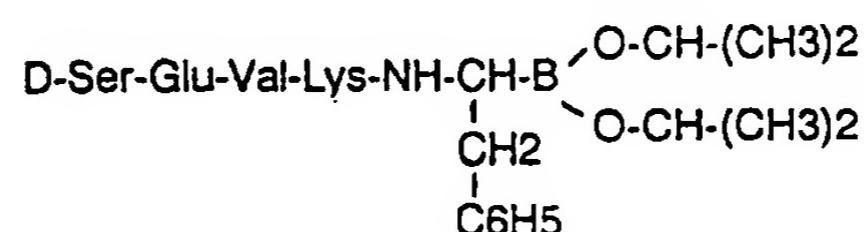
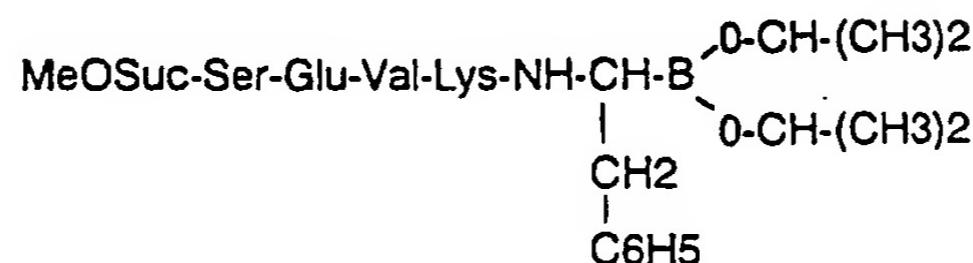
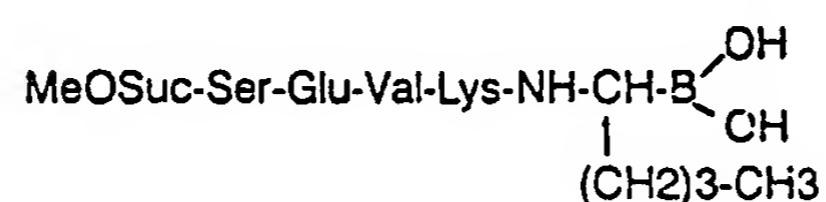
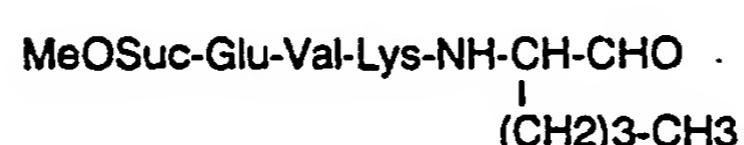
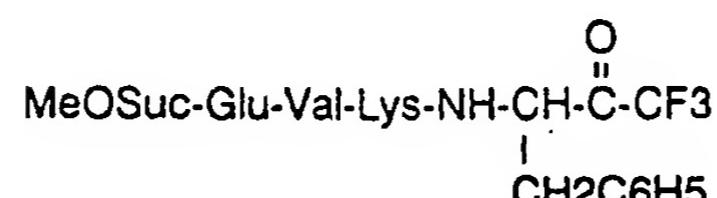
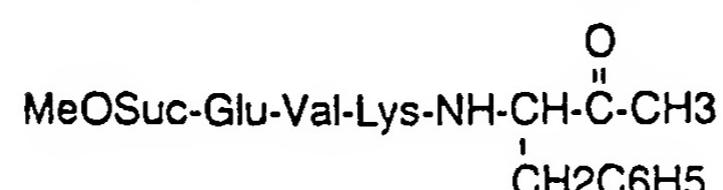
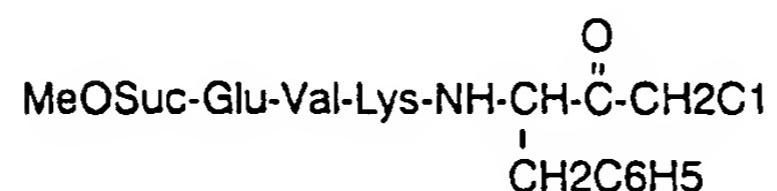
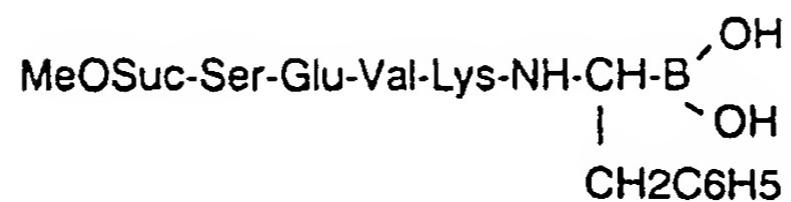
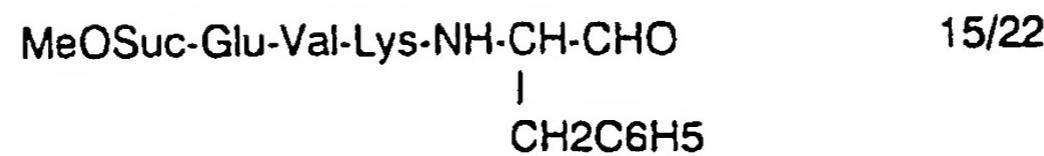


FIG. 16a

SUBSTITUTE SHEET

16/22

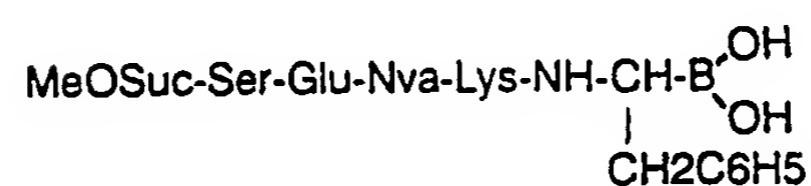
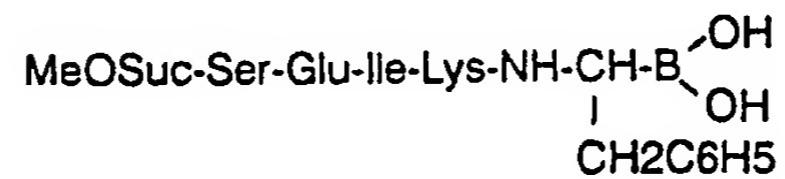
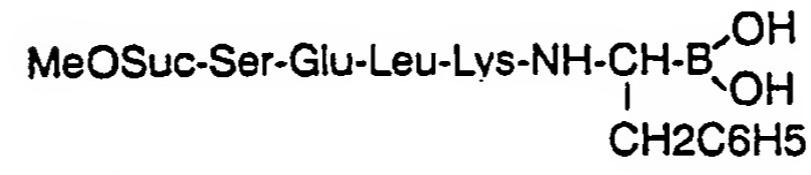
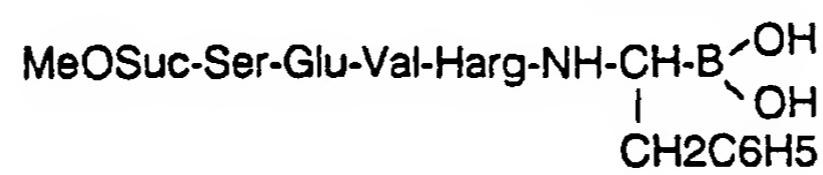
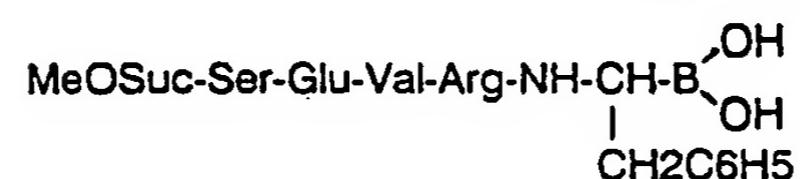
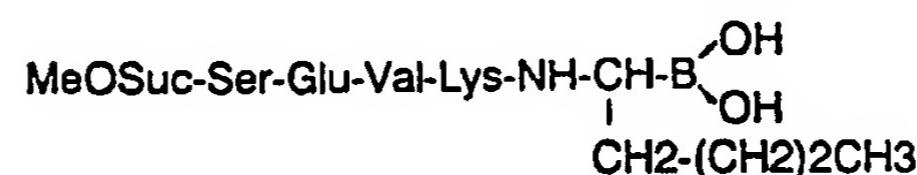
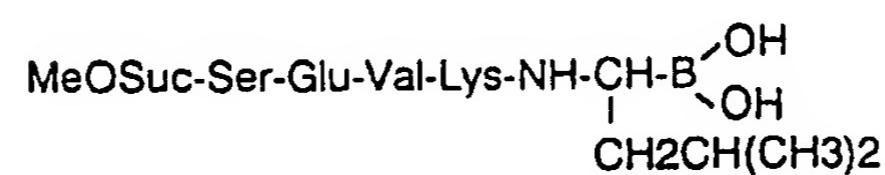
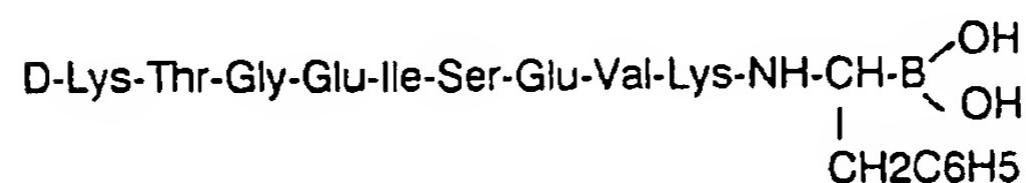
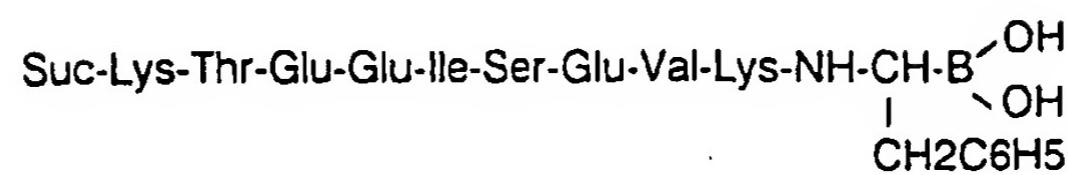
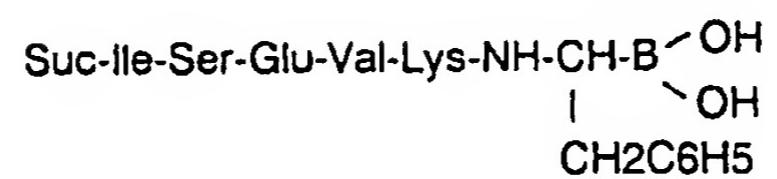
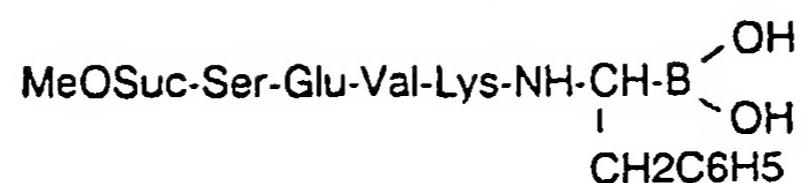


FIG. 16b

SUBSTITUTE SHEE

17/22

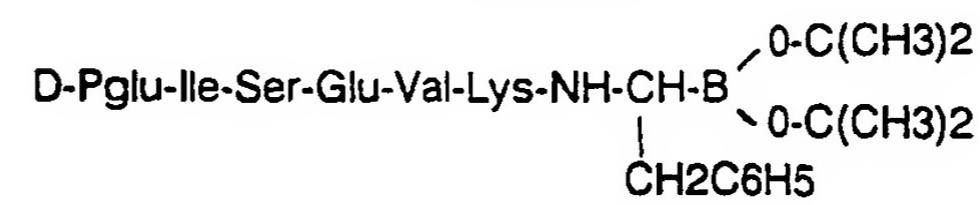
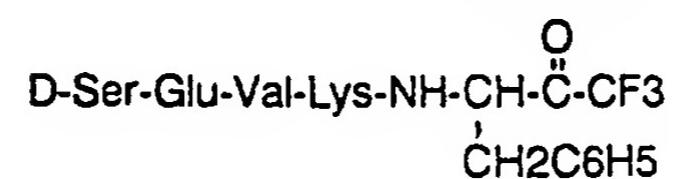
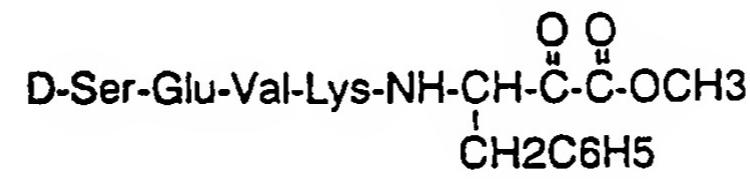
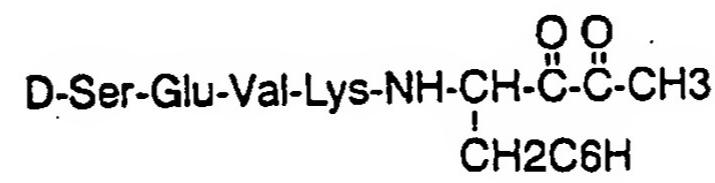
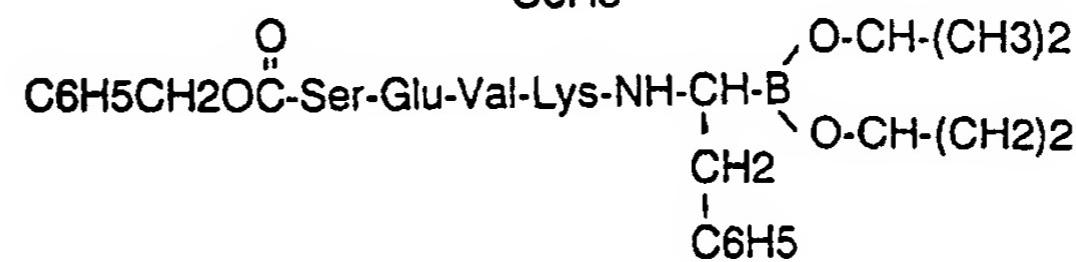
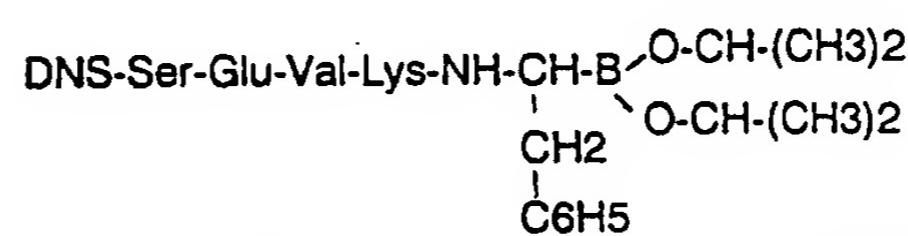
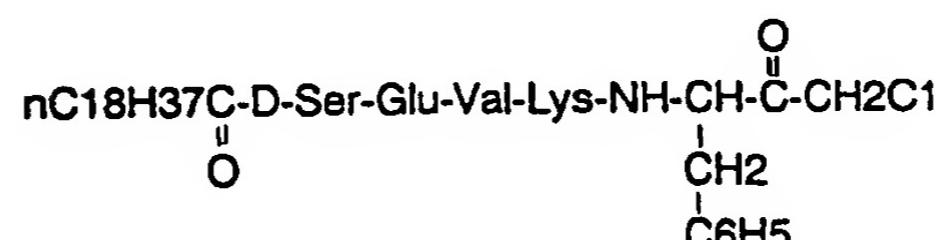
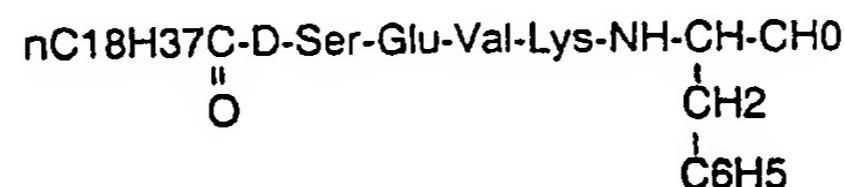
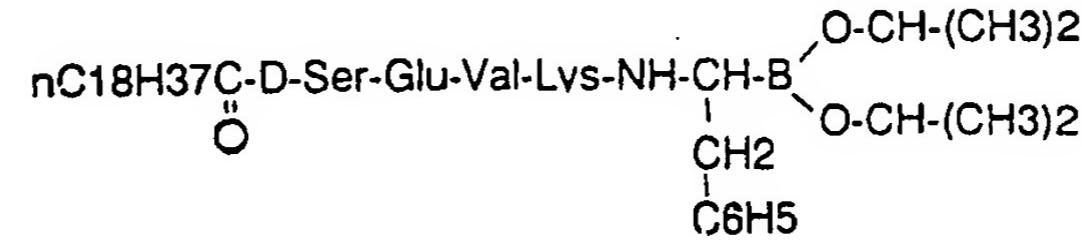
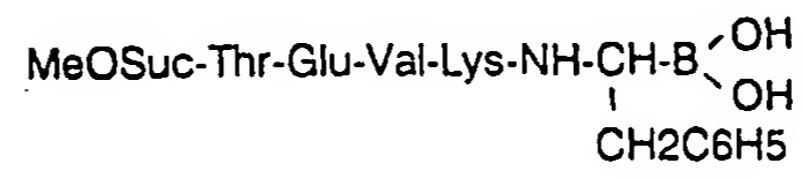
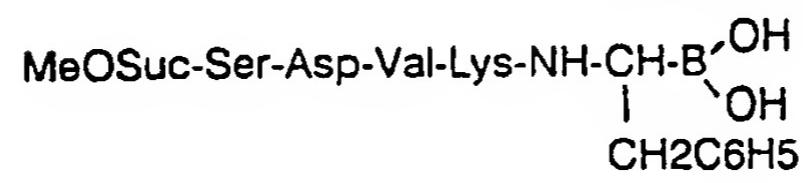
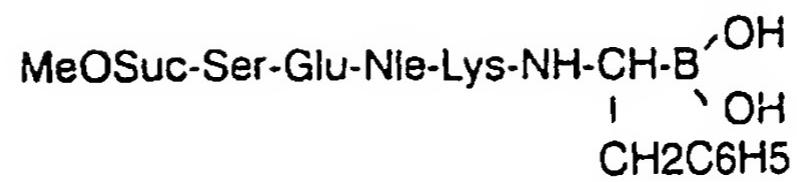


FIG. 16c

SUBSTITUTE SHEET

18/22

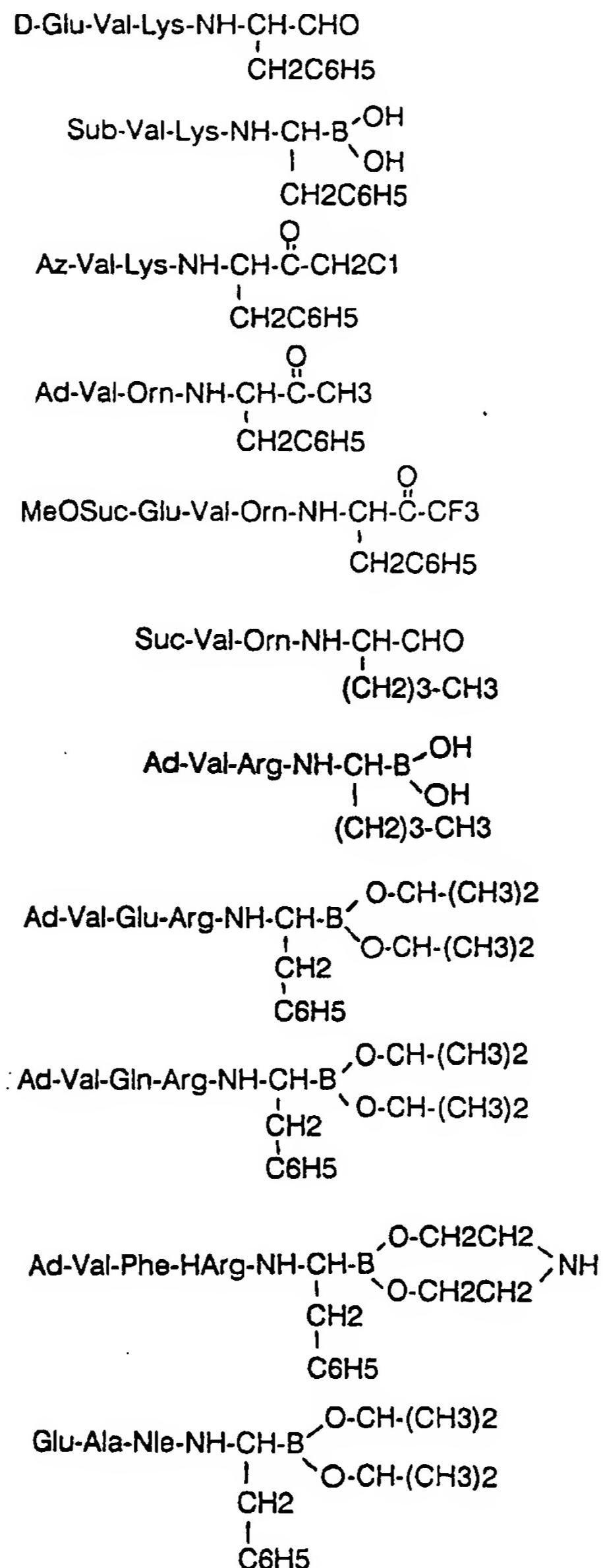


FIG. 16d

SUBSTITUTE SHEET

19/22

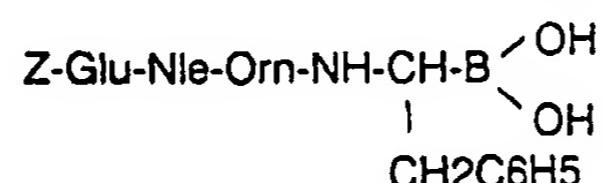
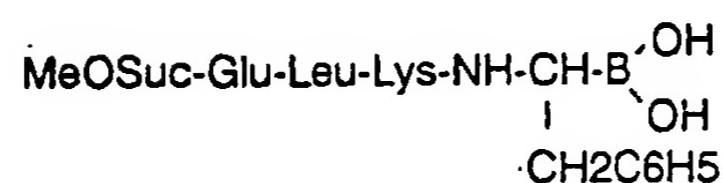
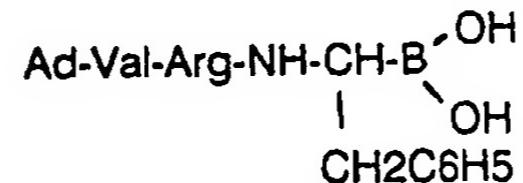
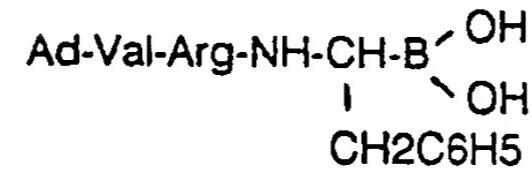
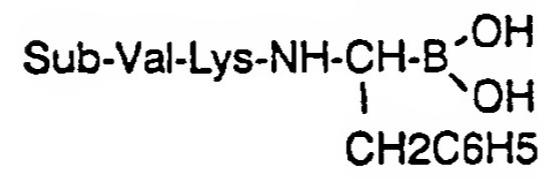
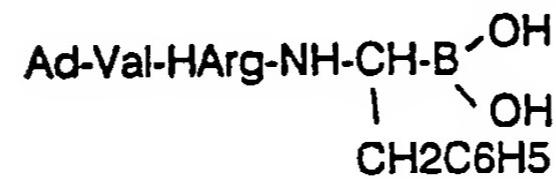
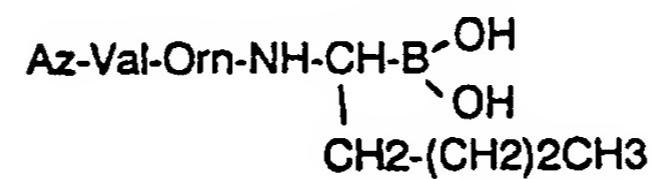
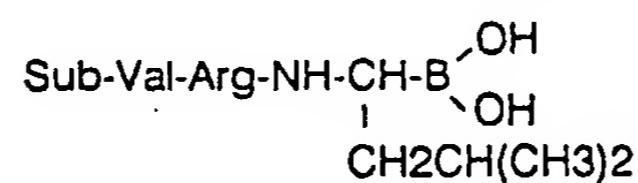
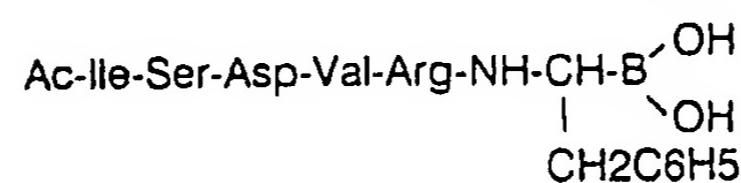
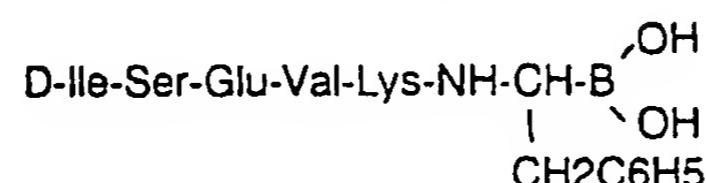
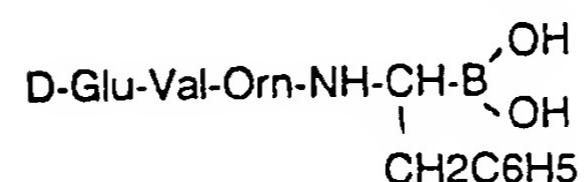
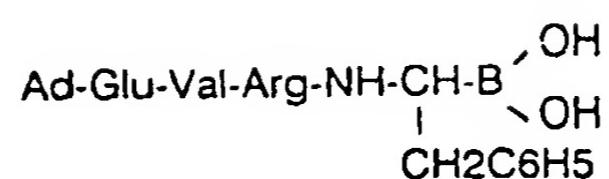


FIG. 16e

SUBSTITUTE SHEET

20/22

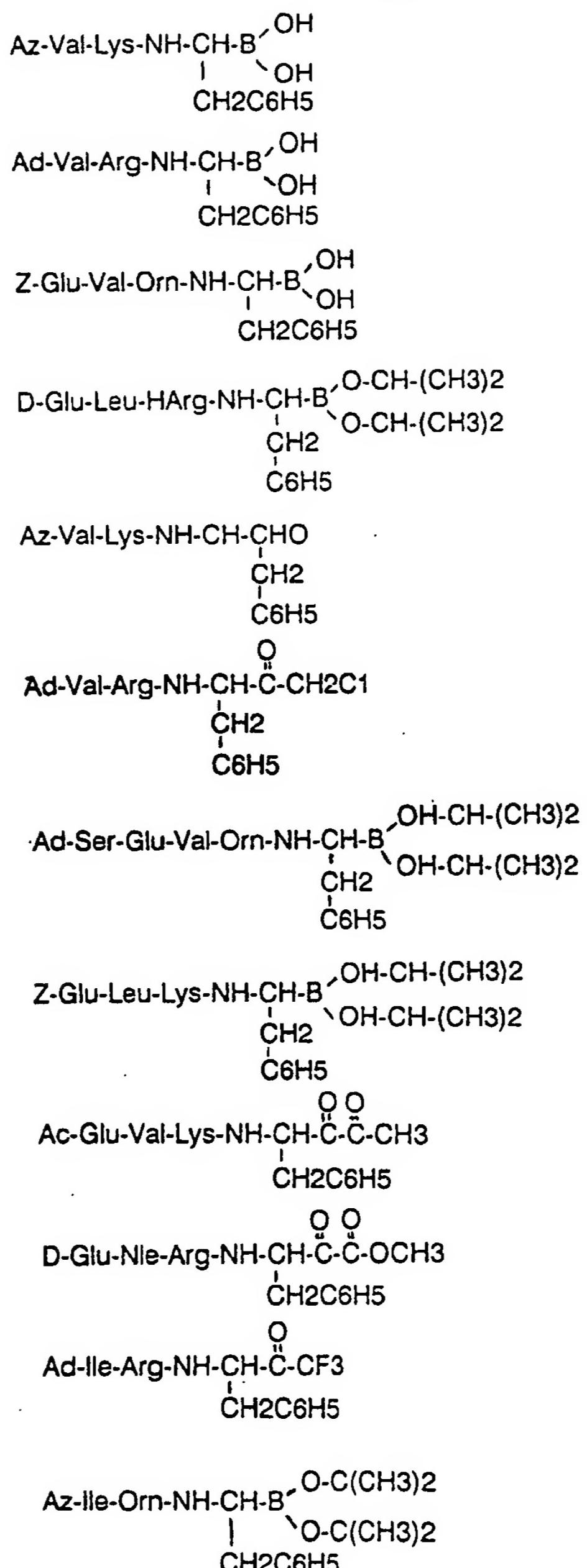


FIG. 16f

SUBSTITUTE SHEET

21/22

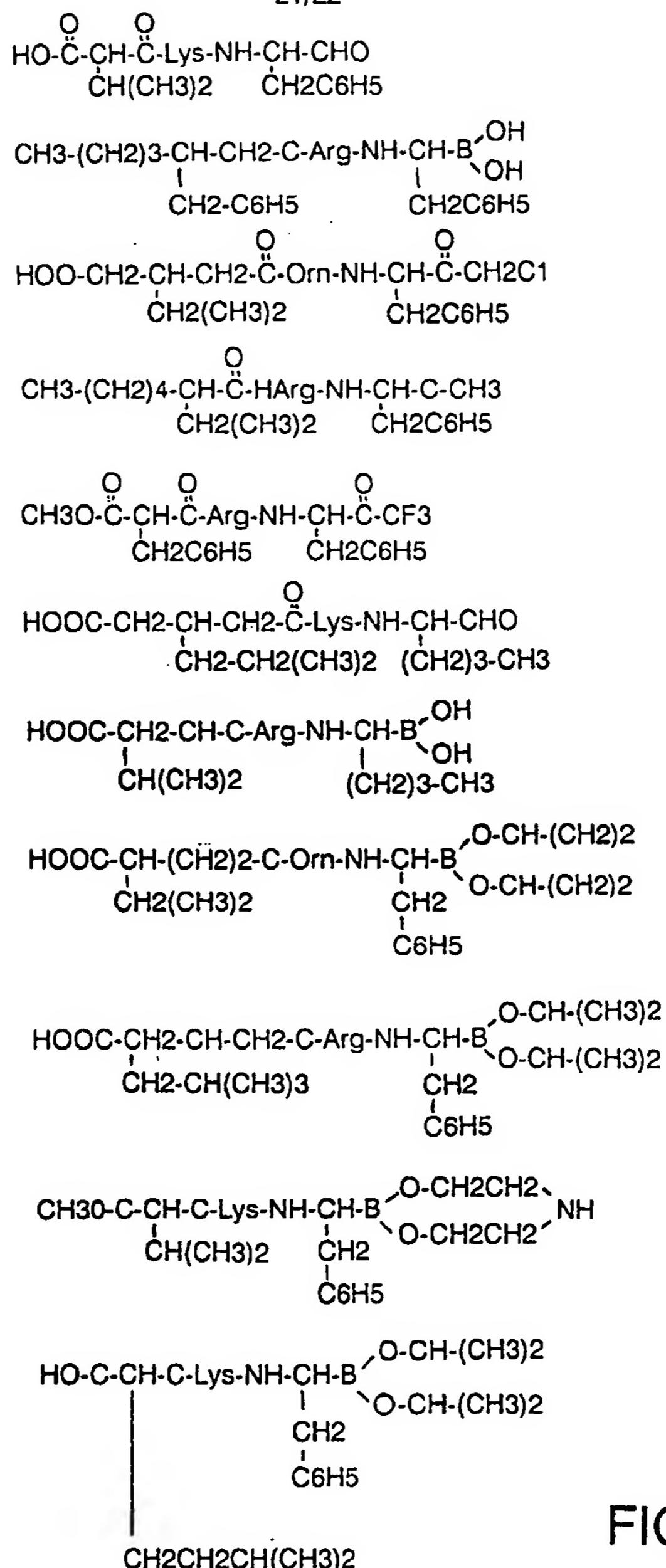


FIG. 16g

SUBSTITUTE SHEET

22/22

MeOSuc-Glu-Val-Lys-AzaPhe-OCH₃

Ad-Val-Arg-AzaPhe-OC₆H₅-NO₂

Sub-Val-Orn-AzaPhe-NH₂

Z-Glu-Val-Lys-AzaPhe-Glu-NH₂

Ad-Phe-Arg-AzaPhe-Asp-OH

Sub-Phe-Orn-AzaPhe-Asp-Ala-NH₂

MeOSuc-Val-Lys-AzaPhe-Asp-Ala-Glu-Phe-OH

Z-Glu-Val-Phe-AzaLeu-OCH₃

Ad-Val-Lys-AzaLeu-Asp-Ala-Glu-NH₂

Sub-Phe-Lys-AzaMet-Asp-Ala-NH₂

Ac-Glu-Val-Arg-AzaNle-Asp-Ala-NH₂

Ad-Val-Orn-AzaNle-Asp-NH₂

FIG. 16h

SUBSTITUTE SHEET